PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification [7]: C07K 16/00, 16/02, A61K 48/00, C12Q 1/68, G01N 33/577, C12N 15/70, 15/85, 15/867

A1

(11) International Publication Number: **WO 00/29444**

(43) International Publication Date: 25 May 2000 (25.05.00)

(21) International Application Number: PCT/US99/26843

(22) International Filing Date: 12 November 1999 (12.11.99)

(30) Priority Data:
60/108,487 16 November 1998 (16.11.98) US

(71) Applicant *(for all designated States except US)*: GENWAY BIOTECH, INC. [US/US]; Welsh Commons, Suite E2, 1364 Welsh Road, N. Wales, PA 19454 (US).

(71)(72) Applicant and Inventor: DUAN, Lingxun [US/US]; 130 Gwynedd Lea Drive, N. Wales, PA 19454 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: GENERATION OF ANTIBODIES USING POLYNUCLEOTIDE VACCINATION IN AVIAN SPECIES

(57) Abstract

The present invention relates to a process for producing antibodies to an antigen in an avian species using polynucleotide vaccination. The present invention also relates to a process for determining the proteomics profile of a set of pre–selected DNA sequences isolated from a bio–sample, preferably the proteomics profile of a human cDNA library. The present invention further relates to a process for identifying physiologically distinguishable markers associated with a physiologically abnormal bio–sample.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

GENERATION OF ANTIBODIES USING POLYNUCLEOTIDE VACCINATION IN AVIAN SPECIES

This application claims the benefit of priority under 35 U.S.C. §119(e) to U.S. provisional application Serial No. 60/108,487 to Linxun Duan, filed November 16, 1998, and entitled METHODS AND VECTORS FOR GENERATING ANTIBODIES USING POLYNUCLEOTIDE VACCINATION IN AVIAN SPECIES.

1. FIELD OF THE INVENTION

The present invention relates to a process for producing polyclonal and monoclonal antibodies to an antigen in an avian species, preferably in a chicken, using polynucleotide vaccination. The present invention also relates to a process for determining the proteomics profile of a set of pre-selected DNA sequences isolated from a bio-sample, preferably the proteomics profile of a human cDNA library. The present invention further relates to a process for identifying physiologically distinguishable markers associated with a physiologically abnormal bio-sample.

2. BACKGROUND OF THE INVENTION

2.1. POLYNUCLEOTIDE VACCINATION IN CHICKEN

Genetic immunization represents a novel approach to vaccine immune therapeutic development. In comparison with the conventional vaccination, genetic vaccination has the advantages of relatively short development time, ease of large-scale production, low development, manufacturing and distribution costs, and better safety for the vaccine producers, administers and receipts.

Genetic vaccination can be divided into DNA vaccination and mRNA vaccination. Recent studies have revealed the following important features of plasmid DNA immunization (Chattergoon et al., *FASEB*, 1997, 11:753-763). First, different tissues, based on the delivery method (in particular, the muscle and skin), can be transfected *in vivo* by plasmid DNA and serve as productive antigen factories. Second, protective cellular and humoral responses can be induced through a variety of

delivery methods in some model systems. Third, only small quantities of plasmid DNA are necessary for antigenic stimulation. The success of plasmid DNA immunization in inducing immune responses to several target antigens through several immunization sites and via several unique delivery techniques solidified the concept of DNA vaccines. This technology has since been applied to many disease models including influenza B, hepatitis B virus, malaria, tuberculosis, SIV and HIV type 1 and various cancers (Id.).

Polyclonal antibodies have traditionally been produced in mammals such as mice, rabbits, sheep, goats, and pigs. The antibodies are obtained from the serum after an immunization period. This technique is invasive, time consuming and costly, involving restraint of and blood sample collection from the animals. In contrast, polyclonal antibody production in chickens, especially with the egg yolk as the antibody source, is a non-invasive technique. The concentration of immunoglobulin in egg yolk may be similar to that of serum (Altchul et al., *Nature Genetics*, 1994, 6:119-129). In addition, poultry have a lower phylogenetic status than mammals (European Community Directive 86/609 Article 7), and it is therefore desirable to use birds instead of mammals.

It can be difficult to raise antibodies of high specificity against well conserved mammalian proteins. Because of the large evolutionary distance from mammals, chickens can often be used as antibody producers (Burke et al., *Science*, 1987, 236:806-812). Another advantage of chickens as antibody producers is that the chicken antibodies are often useful in assays of the analogue to the antigen present in other species (Bonaldo et al., *Genome Research*, 1996, 6:791-806; Buckler et al., *Proc. Natl. Acad. Sci.*, 1991, 88:4005-4009).

Use of chickens for production of antibodies on a large scale is associated with obvious advantages. The cost of keeping chickens is similar to or lower than that of rabbits, by tradition the most popular species for polyclonal antibody production. Thus there is an economic advantage in such a replacement, in addition to the lower number of animals needed. When eggs are used as the antibody source,

productivity is much higher than that in mammals. Chickens are obtainable in inbred strains, thus minimizing the genetic variation in antibody response, a problem commonly seen in rabbit antibody production (Bussey, *Yeast*, 1997, 13(16):1501-1503). No technical assistance is necessary for collection and marking of eggs.

Fynan et al., *Proc. Natl. Acad. Sci.*, 1993, 90:11478-11482, described DNA vaccination of mice and chicken using purified DNA expressing an influenza hemagglutinin glycoprotein. Fynan et al. found that 67-95% of the test mice and 25-63% of test chickens were protected against a lethal influenza challenge. Protections occurred in both mice and chicken that did not have detectable levels of anti-influenza antibodies before challenge. Before challenge, the DNA vaccination and booster inoculations raised non-detectable or very low level of anti-influenza antibodies in mice. No data concerning antibody response in chicken after the DNA vaccination were described.

Kodihalli et al., *J. Virol.*, 1997, 71(5):3391-3396, show that gene gun delivery of DNA encoding an H5 HA protein confers complete immune protection to chickens challenged with lethal H5 viruses. However, within the first 3 weeks post-immunization, no detectable antibodies were found in any of the vaccine groups. In addition, no antibodies to any of the three H5 virus antigens were found following booster immunization of the DNA vaccine group before challenge.

2.2. GENOMIC AND PROTEOMIC RESEARCH

According to databases based on expressed sequence tags (ESTs), the human genome consists of about 60,000—100,000 genes, scattered among 3—4 billion nucleotides of chromosome-based DNA code, the sequencing of which could be completed as early as 2005 (James, *Biochem. Biophys. Res. Comm.*, 1997, 231:1—6). However, DNA sequence information provides only a static snapshot of all the possible ways a cell might use its genes. Therefore, this enormous amount of static DNA sequence information needs to be correlated with dynamic information about gene products and their interactions in order

to provide meaningful insight for fundamental biological processes and applications of such insight into various fields.

The word *proteome* was first introduced in July 1995 and was defined as the "total protein complement of a genome" (Wasinger et al., *Electrophoresis*, 1995, 16:1090—1094). Proteomics aims to supplement gene sequence data with information on what proteins are being made where, in what amounts, and under what conditions (Persidis, *Nature Biotechnology*, 1998, 16:393-394). It aims to show how protein cascades inside cells change as a result of specific diseases, thereby identifying novel potential drug targets. It then aims to validate particular drug leads against those targets by providing information on how those leads affect the proteome cascades (Persidis, *Nature Biotechnology*, 1998, 16:100-101). Therefore, in addition to providing answers to fundamental questions about the molecular basis of a cell's state at any point in time, proteomics promises to accelerate novel drug discovery through automated analysis of clinically relevant molecular phenomena.

In view of the fast development in the genomic research, proteomic research is lagging behind. For example, the proteomic characteristics, including the existence, quantity, cellular location and tissue or developmental expression specificity, of the majority of the proteins putatively encoded by the presently known human DNA sequences have not been characterized. Although the currently available large-format 2-DE is capable of producing gels containing up to 10,000 distinct protein and peptide spots, over 95% of the spots separated by such 2-DE gel cannot be sequenced because they are beyond the limits of current high-sensitivity Edman sequencing technology (Persidis, *Nature Biotechnology*, 1998, 16:393-394).

Specific antibodies, if available, are powerful tools for proteomic research. Antibodies are conventionally generated by protein or peptide vaccination of mammals such as mice, rabbits, rats or sheep. However, such vaccination is time consuming and costly. Therefore, due to the vast number of the known DNA sequences to be characterized, it is virtually impossible to use the conventional protein or peptide vaccination technology to generate antibodies for large-scale proteomic research.

Given the great interest in the proteomic research and the usefulness of antibodies in the proteomic research, there is a great need for a fast and economically feasible method for generating antibodies to be used in large-scale proteomic research. The present application addresses this and other needs in the art.

Citation of the references hereinabove shall not be construed as an admission that such references are prior art to the present invention.

3. SUMMARY OF THE INVENTION

The invention described herein encompasses a process for producing antibodies to an antigen in an avian species, which comprises: 1) delivering to said avian species a DNA sequence encoding said antigen operatively linked to a promoter, said promoter being capable of directing expression of said antigen in said avian species, or a mRNA sequence encoding said antigen, in a amount sufficient to induce detectable production of said antibodies to said antigen; and 2) recovering said antibodies from said avian species. Preferably, the avian species being vaccinated is a chicken and the antibodies are recovered from egg yolk of the chicken.

The present invention also encompasses a process for producing an monoclonal antibody to an antigen in a chicken, which comprises: 1) delivering to said chicken a DNA sequence encoding said antigen operatively linked to a promoter, said promoter being capable of directing expression of said antigen in said avian species, or a mRNA sequence encoding said antigen, in a amount sufficient to induce detectable production of said antibodies to said antigen; 2) removing at least a portion of antibody-producing cells from said chicken; 3) immortalizing said removed antibody-producing cells; 4) propagating said immortalized antibody-producing cells; and 5) harvesting the monoclonal antibody produced by said immortalized antibody-producing cells. Preferably, the chicken antibody-producing cells are immortalized by fusing with cells of a chicken B lymphoblastoid cell line or by oncogene transformation.

The present invention additionally encompasses a vector for expressing genes in avian and bacterial cells, which comprises the plasmid depicted in Figures 3A & 3C; and a vector for immortalizing chicken antibody-producing cells, which comprises the plasmid depicted in Figure 12.

The present invention further encompasses a process for determining the proteomics profile of a set of pre-selected DNA sequences isolated from a bio-sample, which comprises: 1) cloning each of said DNA sequences into a dual-expression vector that is capable of expressing said DNA sequences in avian and bacterial cells; 2) delivering said DNA sequence in said dual-expression vector formed in step 1) to an avian species in a amount sufficient to induce detectable production of antibodies to an antigen encoded by said DNA sequence, and recovering said antibodies from said avian species; 3) delivering said DNA sequence, which is delivered to said avian species in step 2), to bacterial cells, and recovering proteins or peptides encoded by said DNA sequence from said bacterial cells; 4) conducting immunoreactions between said antibodies recovered in step 2) with said proteins or peptides recovered from step 3) to validate the immunospecificity of said antibodies; and 5) conducting immunoreactions between said antibodies recovered in step 2) with said bio-samples to determine the proteomics profile of said set of pre-selected DNA sequences. Preferably, the set of pre-selected DNA sequences is a human cDNA library.

Finally, the present invention encompasses a process for identifying physiologically distinguishable markers associated with a physiologically abnormal bio-sample, which comprises: 1) determining proteomics profile of said physiologically abnormal bio-sample through the above described process; 2) determining proteomics profile of a comparable physiologically normal bio-sample through the above-described process; and 3) comparing the proteomics profile obtained in step 1) with the proteomics profile obtained in step 2) to identify physiologically distinguishable markers associated with a physiologically abnormal bio-sample.

4. BRIEF DESCRIPTIONS OF THE DRAWINGS

Figure 1 depicts a method for selecting DNA clone of interest.

Figure 2 depicts a diagram of the antibody assisted method for identification of gene and protein (AMIGAP).

Figure 3A depicts restriction map of pS&DV; Figure 3B depicts the construction pS&DV; Figure 3C depicts restriction map of pS&DV-S; and Figure 3D depicts the construction pS&DV-S.

Figure 4 illustrates potential immune response elicited by DNA vaccination.

Figure 5 depicts ELISA titering of antibody produced in chicken by DNA vaccination with three antigens encoded by pCMV-HBx, pCI-HBV-pol and pZeoSV2-hCD34.

Figure 6 depicts a restriction map of HbxAg antigen specific expression vector pCMV-HBx.

Figure 7 depicts a restriction map of Hepatitis B virus Polymorantz antigen specific expression vector pCI-HBV-pol.

Figure 8 depicts a restriction map of human CD34 antigen specific expression vector pZeoSV2-hCD34.

Figure 9 depicts binding affinity of IgY produced by DNA vaccination with HBxAg.

Figure 10 depicts SDS-PAGE analysis of IgY purified from egg yolks.

Figure 11 depicts Western Blot analysis of anti-HBxAg IgY produced by DNA vaccination.

Figure 12 depicts restriction map of pImmo vector which can be used for immortalizing chicken B cells.

Figure 13 illustrates a prototype of antibody-chip and its operating procedures.

5. DETAILED DESCRIPTION OF THE INVENTION

Despite the previous failures of generating antibodies by DNA vaccination in chicken, applicant has discovered surprising that polynucleotide vaccination can be used to generate desired antibodies in an avian species. Accordingly, the present invention encompasses processes for producing desired antibodies in an avian species using polynucleotide vaccination, processes for determining the proteomics profile of a set of pre-selected DNA sequences isolated from a bio-sample and processes for identifying physiologically distinguishable markers associated with a physiologically abnormal bio-sample using the antibodies generated by the polynucleotide vaccination of an avian species.

For clarity of disclosure, and not by way of limitation, the detailed description of the invention is divided into the subsections which follow.

5.1. PROCESSES FOR PRODUCING ANTIBODIES IN AN AVIAN SPECIES BY POLYNUCLEOTIDE VACCINATION

The present invention provides a process for producing antibodies to an antigen in an avian species, which comprises: 1) delivering to said avian species a DNA sequence encoding said antigen operatively linked to a promoter, said promoter being capable of directing expression of said antigen in said avian species, or a mRNA sequence encoding said antigen, in a amount sufficient to induce detectable

production of said antibodies to said antigen; and 2) recovering said antibodies from said avian species.

In a preferred embodiment, the avian species to be vaccinated is selected from the group consisting of a chicken (*Gallus*), a turkey (*Meleagris gallopavo*), a duck, a goose and a Japanese quail (*Coturnix japonica*). More preferably, the avian species to be vaccinated is a chicken.

Examples of other names of chicken include, but are not limited to, *Gallus* (*G. domesticus*), chick and hen. Such synonyms are encompassed by the present invention. For consistency, and not for limiting the scope of the presently claimed invention, only the name "chicken" is used herein.

The DNA or mRNA sequence can be delivered to the interstitial space of tissues of the animal body, including those of muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular, fluid, mucopolysaccharide matrix among the reticular fibers or organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation of the lymph fluid of the lymphatic channels.

The DNA or mRNA sequence can be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression can be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts.

In a specific embodiment, the DNA or mRNA sequence is delivered directly to a tissue of the avian species. Preferably, the DNA or mRNA sequence is delivered directly to muscle, skin or mucous membrane. Delivery to the interstitial space of muscle tissue is preferred

because muscle cells are particularly competent in their ability to take up and express polynucleotides.

The DNA or mRNA sequence can be delivered directly to a tissue of the avian species by injection, by gene gun technology or by lipid mediated delivery technology. The injection can be conducted via a needle or other injection devices. The gene gun technology is disclosed in U.S. Patent No. 5,302,509 and the lipid mediated delivery technology is disclosed in U.S. Patent No. 5,703,055, the contents of which are incorporated herein by reference.

In still another specific embodiment, the DNA or mRNA sequence is delivered to a cell of the avian species and said cell containing the DNA or mRNA sequence is delivered to a suitable tissue of the avian species. Preferably, the DNA or mRNA sequence is delivered to a blood cell of an avian species. More preferably, the DNA or mRNA sequence is delivered to a spleen B cell of an avian species.

The DNA or mRNA sequence can be delivered to the cells of an avian species by a number of methods (*see generally* Koprowski & Weiner, DNA vaccination/ genetic vaccination, 1998. Springer-verlag Berlin Heidelberg) including $Ca_3(PO_4)_2$-DNA transection (Sambrook et al., *Molecular Cloning*, 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press, 1989), DEAE dextran-DNA transfection (Sambrook et al., *Molecular Cloning*, 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press, 1989), electroporation (*e.g.*, protocols from Bio-Rad), transfection using "LIPOFECTIN"™ reagent (*e.g.*, protocols from BRL-Life Science), gene gun technology (U.S. Patent No. 5,302,509), or viral gene delivery system (Kaplitt et al., Viral Vectors, Academic Press, Inc., 1995).

The gold-particle based gene gun delivery is the preferred method for delivering the DNA or mRNA sequences (U.S. Pat. No. 5,302,509). In a specific embodiment, Bio-Rad helios gene gun system is used in the DNA vaccination procedure. (BIO-RAD Inc. New England). The helios gene gun is a convenient, hand-hold device that provides rapid and direct gene transfer *in vivo*. The device employs an adjustable, helium pulse to sweep DNA coated gold microcarriers from the inner wall of a small plastic cartridge directly into the target cells. The tubing

prepstation and tubing cutter provide a simple way to prepare 50 cartridge "bullets" at a time.

In still another specific embodiment, a DNA sequence encoding the antigen operatively linked to a promoter, which is capable of directing expression of the antigen in the avian species, is delivered. Preferably, the DNA sequence to be delivered is a plasmid.

The promoter to be used can be an endogenous promoter of the avian species. Alternatively, the promoter can be an exogenous promoter, such as a viral promoter, which is capable of directing expression of the antigen in avian species. Preferably, the viral promoter is RSV LTR, MPSV LTR, SV40 IEP, CMV IEP, metallothionein promoter (U.S. Patent No. 5,703,055) or spleen necrosis virus LTR (SNV LTR) (U.S. Patent No. 5,703,055).

In yet another specific embodiment, the DNA sequence used to vaccinate the avian species further comprises a sequence that directs secretion of the encoded antigen in the avian species. Preferably, the secretion-directing sequence is a leader sequence. More preferably, the leader sequence is an endogenous leader sequence of the avian species such as the leader sequence of VH1 of chicken IgY (Kabat et al., Sequences of Proteins of Immunological Interests, 1983, U.S. Department of Health and Human Services, Washington, D.C.), chicken SPARC (GenBank Accession No. L24906; Bassuk et al., *Eur. J. Biochem.*, 1993, 218(1):117-127), chicken serum albumin (GenBank Accession No. V00381 and J00806; Hache et al., *J. Biol. Chem.*, 1983, 258(7):4556-4564) and chicken tissue-type plasminogen activator (tPA) (GenBank Accession No. U31988). Although endogenous avian leader sequence is preferred, other types of leader sequences can be used in the present invention. In addition to the leader sequence, cell-membrane-directing sequence of any known membrane proteins can used. Examples of such cell-membrane-directing sequence include, but are not limited to, that of IL-1, CD4 and MHC.

In yet another specific embodiment, a mRNA sequence encoding the antigen is delivered.

The polynucleotide material to be delivered according to the present invention can take any number of forms, and the present invention is not limited to any particular polynucleotide coding for any particular polypeptide.

With the availability of automated nucleic acid synthesis equipment, both DNA and RNA can be synthesized directly when the nucleotide sequence is known by a combination of PCR cloning and fermentation. Moreover, when the sequence of the desired polypeptide is known, a suitable coding sequence for the polynucleotide can be inferred.

As disclosed in U.S. Patent No. 5,703,055, when the polynucleotide to be used is mRNA, it can be readily prepared from the corresponding DNA *in vitro*. For example, conventional techniques utilize phage RNA polymerase SP6, T3, or T7 to prepare mRNA from DNA templates in the presence of the individual ribonucleoside triphosphate. An appropriate phage promoter, such as a T7 origin of replication site can be placed in the template DNA immediately upstream of the gene to be transcribed. Systems utilizing T7 in this manner are well known, and are described in the literature, *e.g.*, in Current Protocols in Molecular Biology, §3.8 (vol. 1 1988).

One particularly preferred method for obtaining the mRNA used in the present invention is the use of pXGB plasmid or any similar plasmid that can be readily constructed by those of ordinary skill in the art and can be used with a virtually unlimited number of cDNAs in practicing the present invention (U.S. Patent No. 5,703,055). Such plasmids may advantageously comprise a promoter for a desired RNA polymerase, followed by a 5' untranslated region, a 3' untranslated region, and a template for a poly A tract. There should be a unique restriction site between these 5' and 3' regions to facilitate the insertion of any desired cDNA into the plasmid. Then, after cloning the plasmid containing the desired gene, the plasmid is linearized by cutting in the polyadenylation region and is transcribed *in vitro* to form mRNA transcripts. These transcripts are preferably provided with a 5' cap. Alternatively, a 5' untranslated sequence such as EMC can be used which does not require a 5' cap.

While the foregoing represents a preferred method for preparing the mRNA, it will be apparent to those of skill in the art that many alternative methods also exist. For example, the mRNA can be prepared in commercially-available nucleotide synthesis apparatus. Alternatively, mRNA in circular form can be prepared. Exonuclease-resistant RNAs such as circular mRNA, chemically blocked mRNA, and mRNA with a 5' cap are preferred, because of their greater half-life *in vivo*.

In particular, one preferred mRNA is a self-circularizing mRNA having the gene of interest preceded by the 5' untranslated region of polio virus (U.S. Patent No. 5,703,055). It has been demonstrated that circular mRNA has an extremely long half-life (Harland & Misher, *Development*, 1988, 102:837-852; Pelletier & Sonnenberg, *Nature*, 1988, 334:320-325. This material may be prepared from a DNA template that is self-splicing and generates circular "lariat" mRNAs, using the method of Been & Cech, *Cell*, 1986, 47:206-216. The contents of these articles are hereby incorporated herein by reference.

Also as disclosed in U.S. Patent No. 5,703,055, the present invention includes the use of mRNA that is chemically blocked at the 5' and/or 3' end to prevent access by RNAse. (This enzyme is an exonuclease and therefore does not cleave RNA in the middle of the chain.) Such chemical blockage can substantially lengthen the half life of the RNA *in vivo*. Two agents which may be used to modify RNA are available from Clonetech Laboratories, Inc. Palo Alto, Calif.: C2 AminoModifier (Catalog #5204-1) and Amino-7-dUTP (Catalog #K1022-1). These materials add reactive groups to the RNA. After introduction of either of these agents onto an RNA molecule of interest, an appropriate reactive substituent can be linked to the RNA according to the manufacturer's instructions. By adding a group with sufficient bulk, access to the chemically modified RNA by RNAse can be prevented.

In yet another specific embodiment, a chicken is vaccinated and the antibodies are recovered from egg yolk of the chicken. Preferably, the antibodies are purified from the egg yolk by ammonium

sulfate precipitation, by polyethylene glycol 6000 precipitation or by caprylic acid precipitation.

Methods for recovering antibodies from chicken egg yolk are well known in the art. One such example is disclosed in Svendsen et al., *Laboratory Animal Science*, 1995, 45(1):89-93, the content of which is incorporated by reference. According to Svendsen, eggs are collected daily. The yolk is separated from the white by a domestic egg separator and is washed thoroughly with water to avoid contamination with egg white proteins. The yolk membrane is punctured and the yolk is collected. The yolk is diluted several times with distilled water before storage at -20°C until further purification of the immunoglobulins. To remove yolk lipids, the frozen diluted egg yolk is thawed at room temperature and is centrifuged and filtered to remove the precipitated lipid fraction. This yolk solution is concentrated to the original volume of the yolk by placing the solution in a dialysis tube and removing the water with solid polyethylene glycol 20000.

For ammonium sulfate precipitation, the precipitation is carried out by adding solid AmS to the yolk solution under stirring. To find the optimal precipitation condition, the AmS concentration can be increased stepwise to 50% (*e.g.*, 0 to 10%, 10 to 20%, 20 to 25%, 25 to 30%, 30 to 40%, 40 to 50%). After an incubation period at room temperature, the solution is centrifuged. The pellet is dissolved in phosphate-buffered saline (PBS) containing sodium azide (NaN_3) to avoid microbial growth, and the supernatant is used for the next precipitation step.

For polyethylene glycol precipitation, the precipitation is carried out by stirring solid polyethylene glycol 6000 (PEG) into the yolk solution. To find the optimal precipitation condition, the polyethylene glycol concentration can be increased stepwise to 12% (*e.g.*, 0 to 2%, 2 to 4%, 4 to 6%, 6 to 8%, 8 to 10%, 10 to 12%). After an incubation period at room temperature, the solution is centrifuged. The pellet is dissolved in PBS containing NaN_3. The supernatant is collected and used for the next precipitation step.

For caprylic acid precipitation, the yolk solution is diluted with acetate buffer. Caprylic acid (CA) is stirred into the solution to a final concentration of 0.02, 0.05, 0.1, 0.5, or 1%. After an incubation period at room temperature, the solution is centrifuged. The pellet is dissolved in PBS containing NaN_3.

In yet another specific embodiment, a chicken is vaccinated and the antibodies are recovered from antibody-producing B cells of the chicken, preferably from spleen B cells.

Although the presently claimed processes can be used to generate antibodies to any protein or peptide antigens, the presently claimed processes can preferably be used to generate antibodies to secreted protein or peptide antigens.

In yet another specific embodiment, the present invention provides a vector for expressing genes in avian and bacterial cells, which comprises the plasmid depicted in Figures 3A & 3C, respectively; and a vector for immortalizing chicken antibody-producing cells, which comprises the plasmid depicted in Figure 12.

5.2. PROCESSES FOR PRODUCING MONOCLONAL ANTIBODIES IN CHICKEN USING POLYNUCLEOTIDE VACCINATION

The present invention provides a process for producing a monoclonal antibody to an antigen in a chicken, which comprises: 1) delivering to said chicken a DNA sequence encoding said antigen operatively linked to a promoter, said promoter being capable of directing expression of said antigen in said avian species, or a mRNA sequence encoding said antigen, in a amount sufficient to induce detectable production of said antibodies to said antigen; 2) removing at least a portion of antibody-producing cells from said chicken; 3) immortalizing said removed antibody-producing cells; 4) propagating said immortalized antibody-producing cells; and 5) harvesting the monoclonal antibody produced by said immortalized antibody-producing cells.

Any antibody-producing cells can be removed in step 2) of the above process. In a specific embodiment, the antibody-producing cells are removed from spleen in step 2).

The chicken spleen B cells can be immortalized by any methods known in the art. In a specific embodiment, the chicken spleen B cells are immortalized by fusing with cells of a chicken B lymphoblastoid cell line. Examples of chicken B lymphoblastoid cell line include, but are not limited to, HU3R27, HU3R27N and R27H4. HU3R27, HU3R27N and R27H4 are disclosed in Nishinaka et al., *J. Immunological Methods*, 1991, 139:217-222, the content of which is incorporated herein by reference.

Methods for immortalizing chicken antibody-producing cells are well known in the art and any methods that are disclosed in *Current Protocols in Immunology*, (Ed. Coligan et al.) John Wiley & sons, Inc., 1997 can be used in the present invention. One such example is disclosed in Nishinaka et al., *Journal of Immunological Methods*, 1991, 139:217-222, the content of which is incorporated by reference. According to Nishinaka, chicken B lymphoblastoid cell clones, such as HU3R27, HU3R27N and R27H4, are fused with spleen cells from immunized chickens at a certain parental cell/lymphocyte ratio, *e.g.*, 1:5 at room temperature (RT) with polyethylene glycol 6000 and poly-L-arginine in PBS. The fused cells are gently washed, suspended in IMDM supplemented with FBS and plated in 96-well culture plates at the density of 3×10^5 spleen cells per well based on cell counts before fusion. After 24 h incubation at about 38°C, HAT medium is added to each well, and kept for 10—14 days in the same medium with repeated medium change at intervals of 2—3 days. After 10—14 days, culture supernatants from these wells are used for identification of antibody-secreting hybridomas.

Cloning can be performed by a soft agar culture method. Growing hybridoma cells are distributed to 60 mm plates in soft agar medium containing IMDM, Noble agar (Difco), EBS and conditioned medium from a parental cell culture (Id.). The soft agar plates are allowed to cool at room temperature and then incubated at about 38°C in a CO_2 incubator. Visible colonies are individually removed from the soft agar and adapted to growth in liquid medium.

In still another specific embodiment, the chicken spleen B cells are immortalized by oncogene transformation. Preferably, the oncogene used in transformation is mutant chicken *p53* oncogene or *Ras* oncogene.

5.3. PROCESSES FOR DETERMINING THE PROTEOMICS PROFILE OF A SET OF PRE-SELECTED DNA SEQUENCES ISOLATED FROM A BIO-SAMPLE

In a specific embodiment, the present invention provides a process for determining the proteomics profile of a set of pre-selected DNA sequences isolated from a bio-sample, which comprises: 1) cloning each of said DNA sequences into a dual-expression vector that is capable of expressing said DNA sequences in avian and bacterial cells; 2) delivering said DNA sequence in said dual-expression vector formed in step 1) to an avian species in an amount sufficient to induce detectable production of antibodies to an antigen encoded by said DNA sequence, and recovering said antibodies from said avian species; 3) delivering said DNA sequence, which is delivered to said avian species in step 2), to bacterial cells, and recovering proteins or peptides encoded by said DNA sequence from said bacterial cells; 4) conducting immunoreactions between said antibodies recovered in step 2) with said proteins or peptides recovered from step 3) to validate the immunospecificity of said antibodies; and 5) conducting immunoreactions between said antibodies recovered in step 2) with said bio-samples to determine the proteomics profile of said set of pre-selected DNA sequences.

The set of DNA sequences to be used in the vaccination can be selected according to any criteria or procedures depending on one's interests or purposes to conduct such vaccination. Preferably, the set of pre-selected DNA sequences is a cDNA library.

The cDNA library can be derived from any bio-sample, such as human, animal, plant or microbial sample. Preferably, the bio-sample is of human origin. In addition, the cDNA library can be derived from a bio-sample which is in any physiological state depending on one's interests or purposes to conduct such vaccination.

In a specific embodiment, the present invention provides a method for selecting and constructing a set of DNA sequences to generate antibodies against proteins or peptides encoded by such DNA sequences, which method comprises: (1) selecting specific tissue sample of interest; (2) extracting mRNA from the selected sample; (3) performing cDNA synthesis, preferably using modified RNA normalization procedure; (4) fractionating the synthesized cDNA, preferably by gel electrophoresis; (5) constructing a cDNA library in the vector that can express the cDNA in both avian cells and bacterial cells, such as the pS&DV depicted in Figure 3A and the pS&DV-S depicted in Figure 3C; (6) establishing the fractionated master cDNA library; (7) conducting quality assurance analysis of the master cDNA library; (8) purifying the cloned cDNA; (9) determining the sequence of the cloned cDNA; and (10) conducting bioimformatic analysis of the DNA sequence data to select the set of the DNA sequences for further DNA vaccination. Other criteria and procedures for selecting and constructing an interested set DNA sequences are known in the art and such other procedures are also encompassed in the present invention.

The tissue sample can be obtained from either fresh or frozen sources. The tissue sample can be obtained from human, animal, plant or microbe. Selection of specific tissue will be determined in each specific study. For example, if one desires to obtain human liver specific gene, one could use human adult liver or fetal liver tissue as the resource for mRNA extraction. In a preferred embodiment, the tissue is used as fresh as possible.

The technology for preparation of mRNA is well known in the art. For example, the procedures described in Ausubel et al., *Current Protocols In Molecular Biology*, New York, John Wiley and Sons, 1995; Dracopoli et al., *Current Protocols in Human Genetics*, New York: John Wiley and Sons, 1995; and Sambrook et al., *Molecular Cloning*, 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press, 1989 can be used. Alternatively, commercially available mRNA extraction kits, such as the kit from Life Science BRL (Gaithersburg, MD) and Promega Inc. (Madison, WI), can be used. In a preferred procedure for extracting specific

message RNA, the tissue sample can be rapidly frozen in liquid nitrogen, grounded and resuspend in RNA extraction buffers such as 4M guanidine solution. Alternatively, mRNA can be extracted directly from the tissue using lysis buffer, and the extracted mRNA can be further purified on an ion exchange column, such a the column from Qiagen (Chatsworth, CA).

The following illustrates procedures for obtaining a cDNA library, which contains low level housekeeping genes, using a modified RNA normalization procedure. Large-scale single-pass sequencing of cDNA clones randomly picked from libraries has proven to be a powerful approach to discover genes (Adams et al., *Science*, 1991, 252:1651-1656; Okubo et al., *Nature Genet.*, 1992, 2:173-179). However, ordinary cDNA libraries may contain a high frequency of undesirable "junky" clones that may not only drastically impair the overall efficiency of the approach, but also seriously compromise the integrity of the data that are generated. Among such junky clones are (a) clones that consist exclusively of poly(A) tails; (b) clones that contain very short cDNA inserts; (c) clones that contain nothing but the 3' half of the NotI-oligo(dT)18 primer used for synthesis of first strand cDNA ligated to the adopter; and (d) chimerical clones. To overcome this problem, the classical procedure for normalization and subtraction of RNA for cDNA synthesis can be used (Bonaldo et al., *Genome Research*, 1996, 6:791-806; Neto et al., *Gene*, 1997, 186:135-142). The disadvantage for this procedure is the multiple steps in the manipulation of mRNA resulting in generating the short RNA in the final cDNA products. However, there are alternative procedures that can be utilized. Because a large fraction of all human gene has been identified already, redundant genes which have been characterized from different tissue now can be avoided simply by using biotin-labeled specific redundant gene oligo mixed with oligo dT primer for cDNA synthesis. After finishing the reverse-transcription reaction, the redundant gene cDNA can be removed from the cDNA mixture using avidin-magnetic beads. This procedure can generate cDNA with very low background of housekeeping gene cDNA (Diatchenko et al., *Proc. Natl. Acad. Sci.*, 1996, 93:6025-6030). Another procedure can

also be used to enhance the reverse-transcription reaction of mRNA into cDNA (Gastel & Sutter, *BioTechniques*, 1996, 20:870-875).

The following illustrates procedures for fractionating cDNA by gel electrophoresis. The burden of large-scale DNA sequencing is the repeated sequencing of the same clones multiple times. Using non-amplified cDNA library can help to improve the DNA sequence and checking first-strand cDNA synthesis efficiency, which can also be the index for determining the quality of reverse-transcription reaction (Bodescot & Brison, *BioTechniques*, 1997, 22(6):1119-1125). In a preferred procedure, the total cDNA products can be fractionated by gel electrophoresis, such as 0.8% to 1% agarose gel. Then the desired size of cDNAs can be pooled and extracted before ligating into the cloning vectors, such as the vectors from Qiagen (Chatsworth, CA). The collected length of cDNAs can vary such as every 0.5 kb as the region for the one pooled sample. Those pooled cDNAs can then be inserted into vector in the different ligation tubes designated for different transformation experiments. In the mixed cDNA library, the full-length cDNA often resides in a complex background of small cDNA mixture. This fractionation procedure for cDNA preparation can generate a sub-population of cDNA library. The designated length of cDNA clones will also help to identify the full-length clone from the subpopulation of cDNA once the 3'EST sequence are known and the size of gene transcript is obtained from the standard RNA Northern Blot experiments (Chenchik et al., *Bio/Techniques*, 1996, 21:526-534). This procedure is applicable to the construction a variety of cDNA libraries from different tissue samples.

The following illustrates procedures for constructing cDNA library using a dual-expression vector, such as the pS&DV and pS&DV-S depicted in Figure 3A and 3C, respectively. The purpose for directly cloning cDNA insert into a dual expression vector is to enable conducting DNA vaccination in an avian species and to expressing encoded protein or peptide antigen in bacteria with the same DNA. This dual functional vector carries those fragments for avian cell gene expression such as endogenous avian promoters, or viral promoters such CMV promoter, SV40 intron and SV40 polyadenylation site. The vector also carries T7

RNA polymerase promoter expression system in which the cloned gene will express in *E. coli* strain that carries T7 RNA polymerase such as BL23(DE3) (Studier et al., *Methods in Enzymol.*, 1990, 185:60-89). The cDNA fragments can be directionally inserted into the vector by digesting the cDNA at both ends with different restriction enzymes, such as PacI for 5' and NotI for 3' end. Cloning the cDNA with correct orientation will ensure the expression of the gene. Considering that most of cDNA fragments may lack 5' region or translation initiation code (ATG) in its fragment, the artificial ATG has been created in the vector to enhance the protein expression level in the bacterial by adjusting the distance of Shine-Dalgarno/Kozak consensus sequence between the ATG. There are multiple cloning sites located just downstream of ATG. And three sets of different open read frames (ORF) have been constructed in the vector. In case the cDNA in the vector is not in the desired or correct ORF, it can be easily transferred into the right ORF vector using the restriction enzyme digestion. In addition to all the elements in pS&DV, pS&DV-S contains a chicken IgY leader sequence, which can direct secretion of the proteins or peptides encoded by the cDNA inserts in chicken cells.

The following illustrates procedures for constructing a master cDNA library consisting of subpopulation of fractionated cDNA clones. The vector and cDNA ligation mixture can be efficiently transformed into bacterial cells such as HB101 cells using standard procedure, preferably by electroporation, which are available from different commercial vendors, such as Life Science BRL (Gaithersburg, MD). After plating the transformed bacterial cells on the culture plate and incubating the cells overnight, the clones can be picked up and then transferred into 320 well plate which contains frozen reserve solution such as 15% glycerol (Ausubel et al., *Current Protocols In Molecular Biology*, New York, John Wiley and Sons, 1995; and Sambrook et al., *Molecular Cloning*, 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press). As each fractionated cDNA preparation is pooled as sub-population library, the separated clones for total library should preferably be at least 10 times more than potential human gene numbers. Preliminary data from published data base indicate that there may be approximately 100,000 functional genes

in human genome. So for each tissue-specific cDNA library, about 1 million clones have to be picked up for further DNA sequencing analysis. The automatic clone pick-up systems are available from different commercial vendors such as Stanford University, DNA sequence Center, CA.

The following illustrates procedures for conducting quality assurance analysis of the master cDNA library. Such quality assurance analysis before large-scale DNA sequencing will ensure the desired outcome in a cost efficient fashion. There are several ways in which a portion of cDNA library can be analyzed and then the data be used for determining the quality of the total cDNA library. According to one procedure, about 1,000 clones can be randomly picked up from the library, DNA sequencing can be performed using a specific primer for cDNA 5' sequence. Analyses of such limited sequencing data will give useful information such as the gene distribution pattern, the length of inserted gene and percentage of vector self-ligation. Alternatively, about 2,000 clones can be cultured on a plate(s) and be replicaed to nitrocellulose membrane and be screened by DNA hybridization using housekeeping gene sequence as the probe. For example, if the cDNA library is derived from liver tissue, β-actin and/or albumin nucleotide sequences can be used as the probe. The probes can be labelled by any techniques known in the art. In a preferred procedure, the probe is labelled using the random primer method (Feinberg & Vogelstein, *Analyt. Biochem.*, 1983, 132:6-13; Dracopoli et al., *Current Protocols in Human Genetics*, New York, John Wiley and Sons, 1995). Preferably, ^{32}P-dNTP is incorporated into a random primer labeling reaction using a kit such as the DECprime II DNA labeling kit (Ambion, Austin, TX). Other isotopes such as ^{35}S or ^{33}P can also be used for labelling reaction. Alternatively, nonisotopic labeling agents can be used (Kricka, ed., *Nonisotopic Probing, Blotting, and Sequencing*, 2nd Ed. San Diego, CA, Academic Press, 1995). The hybridization results can be used to review the background contamination of housekeeping genes in the normalized cDNA library. Since most of the housekeeping genes occur at about the same rate in cDNA library generally, the hybridization rate for housekeeping genes can

be used to determine the quality of the cDNA library. The quality of the cDNA library can also be determined by PCR-based procedures. (Pacchioni et al., *BioTechniques*, 1996, 21:644-649). In a preferred procedure, PCR amplifications are carried using the housekeeping genes, such as β-actin gene, as the 3' DNA specific oligo and the T7 promoter oligo as the 5' primer in the randomly picked cDNA library clones. Subsequent detection for presence or absence of PCR products (+/- scores) is carried out either by gel electrophoresis or by internal oligonucleotide hybridization. The PCR amplification results will not only reveal the percentage of the housekeeping gene's presence in the cDNA library, but can also be used to determine the average length of the cDNA insert. The PCR amplification reaction of the random clones of the cDNA library can be conducted using commercially available reagents or kits, such as the ones produced by Origene Technologies, Inc. (Rockville. M.D).

The cloned plasmid DNA can be purified by any methods known in the art. Preferably, the automatic plasmid purification equipment such as the Quiagen Inc automation system 9600 (Valencia, CA) can be used to provide highly purified DNA template for subsequent DNA sequencing analysis. Alternatively, cDNA Clones can be used for PCR amplification and nest-PCR again to provide DNA sequencing template. Since the sequence is known, the two pare of primers for PCR can be easily standardized for all of the clones in the library.

DNA sequences can be determined by any methods known in the art. Preferably, each randomly selected clone is purified from a cDNA library, a DNA sequencing template is prepared. This template is sequenced by the dideoxy method, preferably using an automated DNA sequencer, such as an A. L. F. (Pharmacia Biotech, Piscataway, N.J) or an ABI/373 or ABI/377 (Applied Biosystems, Foster City, Calif). In addition to this "shotgun" phase, in which an initial reading is taken from each clone using a universal primer, a "walking" phase takes additional reading from selected clones by use of custom primers. Complete protocols for these and related sequencing methods are described in Ausubel et al., *Current Protocols In Molecular Biology*, New York, John

Wiley and Sons, 1995; and in Sambrook et al., *Molecular Cloning*, 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press. An efficient design produces small (preferably 18 bp-22 bp) oligonucleotides which can be used as " walking" primer for DNA sequence. The oligonucleotide sequences are generally designed to preferentially detect sequences that are related to the ends of genes in the previous DNA sequence database. This selective bias can be achieved either by manually reading of sequence or by examination of the sequences to be compared. Once designed, these oligonucleotides can be ordered from a DNA synthesis service such as the Research Genetics, (Huntsville, AL). Alternatively, the oligonucleotides can be synthesized on a DNA synthesizer, *e.g.*, on the Applied Biosystems (Foster City, CA).

The DNA sequencing reaction products can be separated by electrophoresis, preferably on polyacrylamide gels using fluorescence detection. Other DNA size separation technologies, such as ultrathin gel slabs (Kostichka et al., *Bio/Technology*, 1992, 10:78-81), capillary arrays (Mathies & Huang, *Nature*, 1992, 359:167-169), and mass spectrometry (Wu et al., *Rapid Commun. Mass Spectrom.*, 1993, 7:142-146), can also be used. DNA sequencing analysis without using gel electrophoresis has also been done by hybridization methodologies (Drmanac et al., *Science*, 1993, 260:1649-1652; Southern et al., *Genomics*, 1991, 13:1008-10017). Another approach is the base addition sequencing strategy (BASS), which uses synchronized DNA polymer construction to determine the sequence of unknown DNA templates (U.S. Patent No. 5,302,509; WO 93/21340; and WO 91/06678).

The sequences of the selected clones by "walking" procedure can be assembled into the complete cDNA sequence of the inserted DNA by matching overlaps. Computer programs are available for these tasks (*e.g.*, Rodger Staden programs, Cambridge, UK; DNAStar, Madison, Wis.). Following sequence assembly, similarity and homology searches can be conducted in relevant sequence databases (*e.g.*, Genbank, Bethesda, Md.; EMBL, Cambridge, UK; Phil Green's GENEFINDER, Seattle, Wash) to identify genes and repetitive elements, to infer function, and to determine the sequence's relation to other parts of

the genome and cell (Gonzalez & Sylvester, *Genome Research*, 1997, 7:65-70).

The above described procedures have been successfully applied to sequencing the genomes of several bacteria (Human Genome Sciences, Gaithersburg, Md.) such as *E. coli* (Plunkerr et al., *Nucl. Acids Res.*, 1993, 21:3391-3398), and higher organisms, such as yeast (Oliver et al., Nature, 1992, 357:38-46), human (Martin-Gallardo et al., *Nature Genet.*, 1992, 1:34-39), mouse (Wilson et al., *Genomics*, 1992, 13:1198-1208.) and *C. elegans* (Wilson et al., *Nature*, 1994, 368:32-38; Sulston et al., Nature, 1992, 356:37-41). The automated sequencing of even large genome regions from mapped cosmid (or other) clones is now routine in several centers (Sanger Center, Cambridge, UK; Washington University, St. Louis, Mo.), with very low error at an average cost of $0.38 – 0.50 or less per base. Specific strategies and protocols for these efforts have been described in Griffin and Griffin, ed. *DNA sequencing: Laboratory Protocols.*, New Jersey, 1992).

The following illustrates procedures for computer-based bioinformatic analyses of the cDNA sequence data. In a preferred procedure, the sequences of the selected clones by universal primer from 5′ of inserted DNA can be firstly analyzed using specific computer program. For example, similarity and homology searches can be conducted (Genbank, Bethesda, Md.; EMBL, Cambridge, UK; Phil Green's GENEFINDER, Seattle, Wash) to identify functionally known genes and un-identified cDNA fragments. The junction DNA sequence between the vector and inserted DNA and potential ORFs can be analyzed, which will help to infer gene function and to determine the sequence's relation to other parts of the genome and cell (Altchul et al., *Nature Genetics*, 1994, 6:119-129). For some newly identified sequences, after "walking" procedure, those new sequences can be assembled into a complete cDNA sequence of the inserted DNA by matching overlaps. Computer programs are available for these tasks (*e.g.*, Rodger Staden programs, Cambridge, UK; DNAStar, Madison, Wis). Following sequence assembly, the full-length cDNA coded putative protein can be further analyzed such as for functional domain searching. The analysis data can be categorized into

computer database. Other experiments, such as looking for the DNA transcription control elements after function of the cDNA is mapped, can also be conducted (Fickett & Hatzigeorgious, *Genome Research*, 1997, 7:861-878).

Once the DNA sequences are selected, the processes described in §§ 5.1. & 5.2. can be used to generate desired antibodies, whether polyclonal or monoclonal ones, against the proteins or peptides encoded by such selected DNA sequences.

In a specific embodiment, the DNA sequences used in the DNA vaccination are also delivered into competent bacteria cells to produce the encoded proteins or peptides, which can be used in characterizing the antibodies generated by the DNA vaccination. Preferably, the bacteria cells are competent *E. coli.* cells. Also, preferably, the dual-expression vector depicted in Figure 3A or 3C is used in delivering the DNA sequence into bacteria cells. When the DNA sequences contained in the vector depicted in Figure 3A or 3C are transformed into bacterial cells, such as BL21(DE3) cells, which carry the RNA 7 polymerase, the proteins encoded by the delivered DNA sequences can be expressed at high level in the presence of an inducer, *e.g.*, IPTG. DNA sequences can be delivered into bacterial cells by any methods known in the art (*e.g.*, Ausubel et al., *Current Protocols In Molecular Biology*, New York, John Wiley and Sons, 1995; and in Sambrook et al., *Molecular Cloning*, 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press). Preferably, commercially available systems for DNA transformation, such as the one from Life Science BRL (Geitesburg. MD), can be used.

Bacterially expressed proteins or peptides can be recovered by any methods known in the art (*e.g.*, Ausubel et al., *Current Protocols In Molecular Biology*, New York, John Wiley and Sons, 1995; and in Sambrook et al., *Molecular Cloning*, 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press). For example, transformed bacterial clones can be picked up and grown in LB culture medium. Before harvesting the bacterial cells, an inducer such as IPTG can be added to induce the protein expression. Bacterial cells can be harvested by centrifugation,

resuspended directly in SDS-PAGE lysis buffer and analyzed by SDS-PAGE using commercially available system, such as the one from Bio-RAD Inc. (Hercules, CA).

The immunoreactions between the antibodies generated by the DNA vaccination and the bacterially expressed proteins or peptides can be analyzed by any methods known in the art (*e.g.*, Ausubel et al., *Current Protocols In Molecular Biology*, New York, John Wiley and Sons, 1995; and in Sambrook et al., *Molecular Cloning*, 2nd Edition, Plainview, N.Y. Cold Spring Harbor Press). Preferably, such immunoreactions are analyzed by immunoblotting. For example, after the SDS-PAGE separation, the proteins and peptides to be analyzed can be transferred onto a suitable membrane, *e.g.*, PVDF membrane, according to the procedures described in Schielen et al., *Journal of Immunological Methods*, 1995, 188:33-41. The immunoblotting reaction can be analyzed by any methods known in the art. Preferably, the immunoblotting reactions are detected by commercially available system, such as the Chemiluminescence detecting system from BIO-RAD (Hercule, CA).

The positive results generated from immunoreaction between the antibodies and the bacterially expressed proteins or peptides only confirm that proteins or peptides are encoded by the DNA sequences isolated from bio-samples. After the antibodies are characterized by the immunoreactions between and the bacterially expressed proteins or peptides as described above, further immunoreactions between the antibodies and the bio-sample, from which the DNA sequences are isolated, can be conducted to determine the proteomics profile of the selected DNA sequences.

If there is uncertain about the reading frame, and no unique ORF could be determined for lack of sequence homology or similarity with known sequences, the DNA fragment can be inserted into three ORFs and Western Blot assay using three different antibodies can be performed.

Any known methods can be used to analyze the immunoreactions between the antibodies and the bio-sample. Preferably, immunoblotting, immunoprecipitation and *in situ* immunostaining are

used. In addition, the antibody-based methods can be used in conjunction with other techniques, such as two-dimensional electrophoresis (2-DE), ultra-sensitive mass spectrometry (MS), and other high-throughout functional screening assays (Persidis, *Nature Biotechnology*, 1998, 16:393-394), in the proteomics studies. The examples of such 2-DE and MS analyses include, but are not limited to, isoelectric focusing followed by mass-based separation (ISO-DALT), non-equilibrium based electrophoresis (NEPHGE), and immobilized first-dimension pH gradients (IPG-DALT) (Humphery-Smith . et al. *Electrophoresis*, 1997 18:1217—1242).

One technology for analyzing the immunoreactions between the antibodies and the bio-sample is tissue immunostaining, which technology is well known in the art (Feitelson & Zern, *Clinics In Laboratory Medicine*, W.B.Saunders Com., 1996). Preferably, cryosected tissue samples are used to perform the immunostaining assay because the tissue sample fixed with this method can preserve the cellular antigen structure. The data from this assay may well represent the cellular protein expression pattern in the tested tissue. Alternative, paraffin fixed tissue sample can be used for antibody immunostaining because this type of tissue fixation preserves the tissue for long time and also can be easily collected from different medical research resources. There are several techniques which can be used to improve the immunostaining sensitivity when using paraffin fixed tissue samples (Lantis et al., *Surgical Endoscopy.*, 1998, 12(2):170-176).

The antibodies generated by the present invention and the information obtained from analyzing the immunoreactions between such antibodies and the bio-sample can used in number of ways. One such use is the generation of an antibody index and the incorporation of such antibody index into the known nucleotide sequence databases.

Recent advance in large-scale genomic sequencing requires more powerful tools for analyzing and interpreting the available DNA sequences. Homology or similarity search programs such as BLAST are very effective and reliable computational tools. New powerBLAST has been developed to enhance the function for this type of computer

analysis program (Zhang & Madden, *Genome Research*, 1997, 7:649-656). In this new powerBLAST program, the search results can be exported to the interactive browser Chromoscope, or formatted as ASCII files, or as HTML pages with links to GenBank, MEDLINE, and other components of Entrez for browsing via the World Wide Web. Both the text and graphical views display the result as multiple alignments of cDNA sequences. Annotated features on the matching sequence are superimposed on the alignment, and this greatly facilitates identification of functional domains in the analyzed sequence. The antibody index generated by the present invention can be automatically linked to each of corresponding cDNA sequence, the Western blot data and tissue immunostaining data can be cross-referenced in the database. The subcellular image generated by the immunostaining with the cDNA derived antibody can be stored, and western blot analysis data can be traced in the database for estimating the size of specific cDNA encoded-protein.

The antibodies generated by the present invention can also be used in the functional analysis of the proteins or peptides encoded by new cDNA sequences. The information generated from cDNA derived antibodies can be categorized into group of functional index (Poustka et al., *Cold Spring Harbor Symp. Quant. Biol.*, 1986, 51:131-139). Through the antibody-based analysis, several types of information can be obtained for a target gene. First, whether the cloned cDNA fragment actually encodes the protein. Secondly, through the tissue immunostaining procedure, one can learn what is correct ORF for this gene. Third, where the gene encoded protein is expressed, tissue distribution pattern and subcellular localization can be determined. The expression level of a specific gene can be determined using very-well documented protein such GAPDH or β-actin as internal control. Based on those leading information for a specific gene, one can design multiple-gene functional assays to further elucidate the cellular function of the gene and understand the relationship of the gene with a specific disease, if the gene is linked to a disease or a disorder.

The DNA sequence provides information about the long-term inherited DNA stored in the nucleus and about the physical linkage of the genes in a genomic context. However, it is also useful to know how these genes are expressed and their cellular localization. Toward this end, as described above, cDNA libraries have been constructed to assess gene expression in particular tissues, and methods such as direct selection have been developed to map these cDNAs relative to a genome (Lovett et al., *Proc. Natl. Acad. Sci.*, 1991, 88:9628-9632). Other methods such as exon trapping are similarly used to measure gene expression and map exons (Buckler et al., *Proc. Natl. Acad. Sci.*, 1991, 88:4005-4009). For functional analysis of a gene, many very well developed techniques and system can be used (Christoffersen, *Nature Biotechnology*, 1997, 15:484-484; Nemotoy, *Japanese Journal of Clinical Medicine*, 1998. 56(1):224-232; Bussey, *Yeast*, 1997, 13(16):1501-1503). The present invention can be used to study the proteomics of such selected DNA sequences.

Mutagenesis is a powerful tool to study a gene's function. The selected gene can be mutated and cloned into the specific vector for generating transgenic animal, such mice, and the phenotype of the transgenic animal can be used in elucidating the target gene's function *in vivo* (Stewart, *Molecular Medicine Today*, 1997, 3(3):93; Hickset al., *Nature Genetics*, 1997, 16(4):338-344). Alternatively, the activity of the specific cDNA encoded protein can be inhibited by a variety of technologies, such as modified oligo antisense inhibition (Milner & Southen, *Nature Biotechnology*, 1997, 15:537-541), target sequence–specific ribozyme inhibition (Duan et al., *Gene Therapy*, 1997, 4:533-543) or single chain antibody (sFv) based intracellular immunization approach (Duan et al., *Proc. Natl. Acad. Sci.*, 1994, 91:5075-5079). The present invention can be used to study the proteomics of the selected cDNA sequences of such knock-out organisms.

In a specific embodiment, the present invention provides a process for determining the proteomics profile of a set of pre-selected DNA sequences isolated from a physiologically normal bio-sample. In another specific embodiment, the present invention provides a process for

determining the proteomics profile of a set of pre-selected DNA sequences isolated from a physiologically abnormal bio-sample. The abnormality of such bio-sample can be permanent or temporary, and can be caused by genetic changes or otherwise. Preferably, the physiologically abnormal bio-sample is obtained from a subject who/that has or is known in the high risk of having any diseases or disorders.

In a specific embodiment, the present invention provides a process for identifying physiologically distinguishable markers associated with a physiologically abnormal bio-sample, which comprises: 1) determining proteomics profile of said physiologically abnormal bio-sample through the above described process; 2) determining proteomics profile of a comparable physiologically normal bio-sample through the above described process; and 3) comparing the proteomics profile obtained in step 1) with the proteomics profile obtained in step 2) to identify physiologically distinguishable markers associated with a physiologically abnormal bio-sample.

this invention will be more completely described by means of the following examples, which are to be considered illustrative and not limitative.

6. EXAMPLES

6.1. Construction of dual functional expression vectors

For constructing pS&DV vector, pDual vector, purchased from STRATAGENE Inc. (La Jolla, CA), was used as template vector for amplification of both CMV/T7 promoter expression cassette. In 100 ul PCR reaction tube, 10 ng pDual plasmid DNA was heated to 95°C for 5 minutes and then mixed with 10 pM of both CMV-1 (5'CACCCTGAATTGACTCTCTTTC3') (SEQ ID NO:1) and PacII-1 (5'ATATGAATTCTTAATTAAGATCTCCATGGTGGCCTCTCCTTC3') (SEQ ID NO:2) oligos using standard PCR reaction (Promega PCR Kit). PCR reaction was performed as follow: 40 cycles at 95°C for 1.45 minutes, 55°C for 1.30 minutes, 72°C for 2 minutes. Finally, the PCR product was further incubated at 72°C for 10 minutes. The Products was named as Fragment-I (0.75kb). In the second tube, 10 ng pDual plasmid DNA was

amplified by PCR using the above-described condition with oligo PacI (5'CGCGGAATTCGCGGCCGCTACCAGGTAAGTGTACC3') (SEQ ID NO:3) and oligo ter-2 (5'CGAGTAGTTTAAACAAAAAACCCCTCAAGTCCCG3') (SEQ ID NO:4). The Product was named as Fragment-II (0.6kb). Both Fragments I and II (1 ug of each) were digested with EcoRI and then gel purified in 1% agarose. After the two fragments were ligated using T4 DNA ligase in 50 ul volume overnight at 16°C; 5 ul of ligation mixture were transferred into a new PCR tube and mixed with oligo CMV-1 and Ter-2 for PCR amplification using the same condition as described above. After the 1.36 kb PCR product was digested with PmeI, this 1.36 kb fragment was inserted into PmeI digested cloning vector pT7*. The final vector was named pS&DV.

For preparing the cloning vector pT7*, the purified pT7Blu(R) plasmid, purchased from Novagen Inc., was used as DNA template for PCR amplification. With 10 ng pT7Blu(R) plasmid, in 100 ul PCR reaction mixture, two oligos T7A and T7B were added into reaction and PCR was performed using the same condition as described above. (T7A: 5'AGATCTGTTTAAACCAGGTGGCACTTTTCGG3' (SEQ ID NO:5) and T7B: 5'AGATCTGTTTAAACAGCTGTTTCCTGTGTGA3' (SEQ ID NO:6)). The 2.1 kb PCR product was then digested with BgLII and gel-purified for self-ligation. The resulting vector was named pT7* which carries two unique PmeI sites.

For constructing pS&DV-S vector which carries chicken IgY Vh1 signal domain (MSPLVSSLLLLAALPGLMAA) (SEQ ID NO:7), two oligos were synthesized as follows: Linker-1: (5'AACCCTCTTCCATGAGCCCACTCGTCTCCTCCCTCCTGCTCCTGGCCG CCCTGCCAGGGCTGATGGCGGCC3') (SEQ ID NO:8), Linker-2: (5'CAGATCCTCTTCGAAGCTAGCGGCCGCGAATTCTTAATTAAGGCCGC CATCAGCCCTGGCAG3') (SEQ ID NO:9). One ug of each of Linker-1 and Linker-2 oligo was mixed in 50 ul PCR reaction condition without Taq DNA polymerase, heated to 94°C for 5 minutes, and then was slowly cooled to room temperature. After adding 2.5 units of Taq DNA polymerase, the PCR reaction was performed using the same condition as above but was only run for 10 cycles. The 105 bp PCR product was

digested with Eam1104-I and inserted into pDual vector's Eam1140-I site. The resulting vector was named pDual-S. Using pDual-S as the DNA template, the 1.5 kb DNA fragment which carries the gene expression cassette was generated by further PCR amplification with the CMV-1 and Ter-2 oligos. After PmeI digestion, the 1.5 kb DNA fragment was inserted into pT7* vector's PmeI site to generate the dual function vector pS&DV-S vector.

6.2. In vivo immune response mechanism illustration elicited by DNA vaccination

The direct intracellular inoculation of DNA expression cassettes leads to the in vivo transfection of host cells. Expression of the plasmid-encoded protein may elicit an immune response. Secreted immunogens are ingested by phagocytosis and presented as peptide-MHC II complex by professional antigen-presenting cells. These cells can provide the primary activation signal, costimulatory ligands, and cytokines necessary to stimulate naïve T cells. Stimulation of Th0 T cell with IL-4 leads to the development of Th2 $CD4^+$ helper T cell, which will secrete cytokine to promoter B cell development, including IL-4, Il-5, Il-6, and IL-10. Stimulation of Th0 cells with the proinflammatory cytokine IL-12 and IFN-γ leads to development of the Th1 $CD4^+$ helper T cell. These cells secrete cytokines that will promoter the development of $CD8^+$ cytotoxic T lymphocytes (Koprowski et al DNA vaccination/ genetic vaccination. 1998. Springer-verlag Berlin heidelberg.).

6.3. ELISA titering of the chicken immunized by particle-mediated DNA delivery experiments

As illustrated in Figure 5, three antigen cDNAs which is driven by CMV or SV40 promoter, respectively, were used for vaccinating chickens. Chicken strain used in this experiment, the Hy-line SC strain, was obtained from Hy-Line Inc. (Dallas Center, Iowa).

For each transfection, 1 ug of vector DNA coated on 0.5 mg of gold microparticles was loaded onto a Kapton macroprojectile as previously described (Williams, et al., *Proc. Natl. Acad. Sci.*, 1991,

88:2726-2730). The DNA was delivered into the target site (chicken back skin) using a handheld, helium-driven ballistic gene gun with equivalent of 200 ng plasmid (Sanford, et al., *Technique*, 1991, 3:3-16). The pressure in the gun was adjusted to 1200 psi. After DNA injection, at different post-injection day, the eggs from the immunized chickens were collected and stored at –4°C and IgY was purified using the protocol described in § 6.8. In this experiment, repeated DNA injection with same amount of plasmid DNA was performed to observe the host immuno-response for antigen.

To purify each of the corresponding antigen, *i.e.*, HbxAg, HBV-pol and CD34 from *E. coli* for assaying the specificity and binding affinity of antibodies derived from the immunized chickens, HbxAg gene was cloned into pET3a (Wu et al, *Cell*, 1990, 63:687-695). The RNase H domain of the HBV polymerase protein was PCR amplified using Pol-1 and Pol-2 oligo, and inserted into pET28a NdeI–HindIII site. As described in the following section, human CD34 cDNA was obtained and inserted into PET 28a vector. All of the pET vectors were purchased from Novagen Inc. Each of the constructs was confirmed by DNA sequencing. Recombinant protein expression was assayed according to the Novagen Kit instruction (*see also* Studier et al., *Methods Enzymol.*, 1990, 185:60-89). Briefly, the constructed plasmids were transformed into BL21(DE3) competent cells, clones were transferred into 3 ml LB medium which contains carbenicillin (Sigma Inc. cat # C1389) or kanamycin, and cultured at 37°C overnight. On second day, the culture was transferred into 500 ml LB medium with selection and shaking at 37°C until OD_{600} reached 0.45. Then 0.2 mM IPTG was added into the bacterial culture and the bacteria cells were cultured for two more hours. Cells were cooled on ice for 5 min and then harvested by centrifugation at 5000 x g for 5 min at 4°C. The cell pellet was washed once with cold PBS buffer. The cells were resuspended in 50 ml Tris-buffer (50 mM Tris-HCl, 2 mM EDTA, pH 8.0) and disrupted by sonication. The sonicated samples were separated into soluble or insoluble (pellets) fractions by centrifugation at 5000 x g for 5 min at 4°C. After the pellet were resuspended in 45 ml Tris-buffer containing 100 ug/ml lysozyme and 5 ml 1% triton X-100, 10

ul of both soluble and insoluble samples were loaded on 12.5% SDS-PAGE (BioRad mini-gel system) with untransformed bacteria samples as control. The gel staining showed that all three bacterially expressed proteins *i.e.*, HbxAg, HBV-pol and CD34, were mainly found in the insoluble fraction.

Urea was added slowly to the 50 ml insoluble fraction to a final concentration of 8 M with stirring at 4°C. After the pellet was completely dissolved, the sample was further centrifuged at 5000 x g for 5 min at 4°C to remove the pellet and supernatant was loaded on a 3 ml His-Bind Resin column for purification of the recombinant proteins using the protocol suggested in the Novagen Inc.'s kit. After the washing steps, the purified protein was eluted from the column with 10 ml 1X strip buffer, which contains 6 M urea. The purified protein sample was transferred into a dialysis tube and was dialyzed against PBS overnight at 4°C in 4 Liter volume. Finally, the protein sample in the dialysis tube was further concentrated into 1 ml volume by Amicon spin concentration column (Amicon Inc, MW cut of: 10,000 dalton). After checking protein concentration, 3 ul of purified protein were separated on 12.5% SDS-PAGE. The purity of the recombinant protein, measured by the density of protein bands using molecular densitometry (Molecular Dynamic Inc), is more than 93%.

Five ug of the purified bacterially produced antigen protein, in 200 ul PBS buffer, were coated on ELISA plate well at 4°C overnight. After coated wells were washed once with BPS, 200 ul 5% BSA solution was added for further coating to block non-specific binding in the assay. Chicken IgY (1 mg/ml) purified by the PEG method after the DNA immunization (see § 6.8) was serially diluted, added into each coated well and incubated at 37°C for 2 hours. After 5-time washing with PBS buffer, the HRP-labeled goat anti-chicken antibody (Sigma Inc., 1:10000 dilution) was added into the well and incubated for 1 hour. After 5-time washing with PBS, the substrate buffer was added and incubated for 15 min with ELISA reading every five minutes.

In Figure 5, each of different bars represents different chickens. In Figure 5A, chicken was immunized with pCMV-HBx vector;

in Figure 5B, chicken was immunized with pCMV-HBV-pol vector; and in Figure 5C, chicken was immunized with pZeoSV2-hCD34. Assay time point is as the following: Preimmune (Pre); or 12 days after boost 1 (B1), Boost 2 (B2) and boost 3 (B3). Analysis of the multiple DNA vaccination host immuno-reaction data shows that 12 days after single DNA injection, chicken specific antibody production already reached the detectable level.

6.4. Construction of the pCMV-HBx expression vector

Construction of the hepatitis B X gene expression vector was performed as the following. Ten ng of pTKHH2 DNA (HBV full-length viral genome dimer plasmid) was mixed with MF18 (MF18: 5'GGAAGCTTGCCGCCATGGCTGCTAGGCTGTGC3') (SEQ ID NO:10) and MF19 (MF19: 5'GTGGAGACGGATTAGTACCATGGCC3') (SEQ ID NO:11) oligo in 100 ul PCR reaction tube. HBV polymerase gene was PCR amplified in the following condition: at 95°C for 1.30 minutes, at 55°C 1.30 minutes and at 72°C for 2 minutes; for a total of 40 cycles. Finally, the PCR product was incubated at 72°C for 10 minutes. The 488 bp PCR product was gel purified and then digested with HindIII and KpnI. The digested HBx fragment was inserted into mammalian expression vector pTTW-1 vector (Condreay et al., *J. Virology*, 1990, 64:3249-3258), which was digested with same enzymes to generate the pCMV-HBx plasmid.

The expression of the HBx protein was tested by transfecting the pCMV-HBx into human hepatocellular carcinoma cell line HepG2 cells. 1×10^6 HepG2 cells were seeded in 10 cm culture dish in 10 ml DMEM medium which was supplemented with 10% fetal calf serum at 37°C in CO_2 incubator overnight. Four hours before the transfection, 10 ml fresh pre-warmed DMEM medium supplemented with 10% fetal calf serum was replaced. Five ug purified pCMV-HBx plasmid were mixed in calcium precipitation mixture according the manufacturer's protocol (Promega Inc.'s calcium transfection kit). After the mixture precipitated at room temperature for 30 minutes, the mixture was slowly dropped into HepG2 cells and cultured for 12 hours. Next day, 10 ml pre-warmed fresh medium was replaced and the cells were cultured for one more day.

Transfected cells were washed with 10 ml cold PBS buffer and cells were collected using rubber policeman in 1.5 ml PBS. After centrifugation, the cell pellet was resuspended in 100 ul H_2O, and 20 ul cell sample was mixed with same volume SDS-PAGE loading buffer and boiled for 3 minutes. The boiled sample was subjected to the max-speed Eppendorf centrifugation for 2 minutes and 5 ul of the supernatant were loaded on 12.5 % gel for SDS-PAGE separation. The extracted cellular protein was demonstrated to be positive for HBxAg expression using Western Blot assay with specific rabbit anti-HBx antibody (1: 800 dilution) (Wu et al., *Cell*, 1990, 63:687-695).

6.5. Construction of Hepatitis B polymerase antigen specific expression vector

Construction of the hepatitis B polymerase (HBV pol) gene expression vector was performed as following. In 100 ul PCR reaction as described in § 6.4, pTKHH2 plasmid DNA template was mixed with oligos MF26 (5'AAGAGCTCGCCACCATGGCCCTATCCTATCAAC3') (SEQ ID NO:12) and HBVpol-2 (5'TCACCTTAAGGTGTTGGAAGGTGGTTTGA3') (SEQ ID NO:13). The 868 bp HBV pol 5' end DNA fragment was gel purified and digested with SalI and EcoRI, then inserted into vector pGEM3Z (Promega Inc.) which was digested with the same enzymes to generate the plasmid pGEM3Zpol-5'. From pTKHH2 Vector, another 1638 bp 3'end of HBV polymerase DNA fragment was PCR amplified using pTKHH2 plasmid mixed with the following oligos, Pol-3 (5'GGCCATGCAGTGGAATTCCACTGCCTTCC3') (SEQ ID NO:14) and Pol-4 (5'AACCAAGCTTCACGGTGGTCTGGATGCAAC3') (SEQ ID NO:15). The PCR product was digested with EcoRI and HindIII. The digested fragment was inserted into pGEM3Zpol-5'EcoRI-HindIII site to generate the 2874 bp full-length HBV polymerase gene. The resulting plasmid was named p3Zpol. The HBV polymerase gene was digested with SacI first and then filled in with Klenow reaction (Sambrook et al., *Molecular Cloning*, Second Edition, Plainview, N.Y. Cold Spring Harbor Press, 1989) to form a blunt end. After digestion with SalI again, the full-length polymerase gene was then inserted into pCI vector (Promega Inc.), which

was digested with MuII, Blunted and then digested with SalI, to generate the pCI-HBV-pol expression vector.

The expression of HBV polymerase protein was also tested by transfecting the pCI-HBV-pol into human hepatoma cell line HepG2 as described in § 6.4. The extracted cellular protein was demonstrated to be positive using Western Blot assay with specific rabbit anti-HBV polymerase peptide antibody (Feitelson et al., *Clinics In Laboratory Medicine*, 1996, W.B.Saunders Com).

6.6. Construction of the pZeoSV2-hCD34 expression vector

In order to obtain human CD34 full-length cDNA for DNA vaccination, CD34 positive cell line KG-1a was used for RNA extraction (Simmons et al., *J. Immunol.*, 1992, 148:267-271). Total RNA was purified from $1X10^6$ cultured KG-1a cells by the technique described in Puissant et al., *BioTechniques*, 1990, 8:148-149 with minor modification. Briefly, $2X10^6$ cultured KG-1a cells were suspended in 5 ml buffer (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sarkosyl, 0.1 M 2-mercaptoethanol, pH 7.0). The following reagents were added, punctuated by vortexing of the tube: 2 M sodium Acetate pH 4.0 (0.5 ml), Phenol (5 ml), and chloroform (1 ml). Following incubation on ice for 15 min, the tubes were centrifuged at 10,000 g (7,000 rpm) for 10 min. Isopropanol (5 ml) was added to the upper phase and incubated on ice for 10 min, followed by centrifugation as describe above. The RNA pellet was dissolved in 1 ml 4 M LiCL and transferred to a microcentrifuge tube. The original tube was rinsed with 0.5 ml LiCl and the pellet was vortexed for 5 min in the combined liquid. RNA was pelleted by centrifugation (10 min), resuspended in 1 ml 4M LiCl and pelleted again. The pellet was thoroughly resuspended in TE/0.5% SDS and extracted with an equal volume of chloroform/isoamyl alcohol (24:1). The aqueous phase was extracted a second time before precipitation of RNA by adding 2 M sodium acetate (0.1 ml) and isopropanol (600 ul). RNA was pelleted and resuspended in water.

One ug of the purified total RNA was used for reverse transcription (RT) reaction with oligo dT_{18} primer, following the

manufacturer's protocol (BRL Life Science Inc., RT kit). Full-length human CD34 cDNA was PCR amplified using oligo hCD34-1 (5'GAAGGATGCTGGTCCGCAGGGG3') (SEQ ID NO:16) and hCD34-2 (5'CACCTAGCCGAGTCACAATTCG3') (SEQ ID NO:17) primers. The PCR reaction was performed at the following condition: at 95°C for 1.30 minutes, at 53°C for 1.30 minutes, at 72°C for 2 minutes; and for a total of 40 cycles. Finally, the PCR product was incubated at 72°C for 10 minutes. The 1.2 kb PCR product was directly inserted into the HincII digested pUC18 vector (Phamacia Inc). The resulting plasmid pUC18-hCD34 was confirmed to contain the full-length hCD34 sequence by DNA sequencing analysis using ABI 373 DNA sequencer and M13 primers.

After digestion of the pUC18-hCD34 with HindIII and EcoRI, the 1.2 Kb hCD34 fragment was gel purified and inserted into mammalian expression vector pZeoSV2+ (Invitrogen Inc.), via the HindIII and EcoRI sites to generate the vector pZeoSV2-hCD34. The expression of the pZeoSV2-hCD34 was confirmed by transfecting it into HeLa cells and immunostaining with mouse anti-human CD34 monoclonal antibody (Pharmingen Inc., CA).

6.7. Enzyme-linked immunoassay of chicken antibody to HbxAg

In this experiment, purified *E. coli* derived HBxAg antigen was used for assaying chicken anti-HBx antibody which was generated from the DNA vaccination as described in § 6.3. Five ug of the purified HbxAg antigen in 200 ul PBS buffer were coated on ELISA plate well (Nalge Nunc Internation., Rockester, NY) overnight. After washing with PBS buffer, 200 ul of 5% BSA were added for further coating the well to block non-specific binding in the assay. Chicken antibody was serially diluted in PBS buffer containing 0.1% bovine serum albumin and incubated in microtiter plate coated with HbxAg antigen for two hours. After 5-time washing with PBS buffer, the HRP-labeled goat-anti-chicken antibody (Sigma Inc., 1:10000 dilution) was added into the well and incubated for 1 hour. After another 5-time washing with PBS, the substrate buffer was added and incubated for 15 min with ELISA reading every five minutes. Values are the mean of duplicate samples. Figure 9

shows that DNA vaccination generated chicken antibody that has very high binding affinity to the HBxAg antigen.

6.8. Purification of chicken IgY from DNA immunized chicken egg yolk

Laying hens from Hyline Inc. (Dallas Center, Iowa) were kept on regular light cycles. From day 20 after the chickens were immunized with single injection of pCMV-HBx vector as described § 6.3, eggs were collected until the tenth egg was collected. IgY was extracted according to the method described in Polson et al., *Immunol. Commun.*, 1980, 9:475-493. Briefly, yolks were separated from the whites and broken by dropping through a funnel into a graduated cylinder. An equal volume of a buffer (0.01 M phosphate, 0.1 M NaCl, and 0.01 % NaN_3, pH 7.5) was added and stirred. Pulverized PEG 6000 (Sigma Inc.) was added to a concentration of 3.5% and stirred until it all dissolved. The protein precipitate formed was pelleted by centrifugation at 13,000 g for 10 min. The supernatant was decanted and filtered through cheesecloth and PEG 6000 was added to bring the final concentration to 12%. The mixture was stirred thoroughly and centrifuged again at 13,000 g for 10 min. The pellet was redissolved to the original yolk volume in 0.01 M phosphate-0.1 M NaCl (pH 7.5) and PEG 6000 was added to 12% for a second precipitation. The supernatant was decanted and the pellet was centrifuged twice to extrude the PEG 6000. This final IgY pellet was dissolved in 50 mM Tris-0.1 mM EDTA-25% glycerol-0.02% NaN_3. (pH 7.9).

For further purification, IgY was purified on DEAE-cellulose by adsorption at 0.015 M KPO_4 (pH 8.0) and eluted with a 0.015-0.3 M KPO_4 (pH 8.0) gradient. Four ug purified samples were separated on 4-20% polyacrylamide gradient gel (BIO-RAD commercial mini-gel) and visualized with silver staining (Sambrook et al., *Molecular Cloning*, Second Edition. Plainview, N.Y. Cold Spring Harbor Press, 1989). The purification results are shown in Figure 10. Lane 1 is IgY purified through PEG precipitation and Lane 2 is IgY purified by DEAE-cellulose. H and L indicates the position of immunoglobulin heavy and light chain, respectively.

6.9. Time course of anti-HBx production in hens determined by immunoblot analysis

Figure 10 shows the time course of host immune response to DNA vaccination. The level of anti-HBx antibody was analyzed using purified *E. coli*-derived recombinant HbxAg antigen. Eight ug per well of the purified *E. coli*-derived recombinant HbxAg antigen (as described § 6.3) were separated on a 12.5% SDS-polyacrylamide gel and the separated proteins were transferred onto a PVDA membrane according to the manufacturer's protocol (Bio-Rad mini-gel kit). Yolk antibodies, purified by PEG 6000 precipitation as described in § 6.8, were diluted 1:1200 in Tris-Buffer saline, and 10 ml purified IgY solution were applied to the PVDA membrane and incubated at 37°C for 2 hours. After 5-time washing with PBS buffer, the membrane was transferred into 20 ml of HRP labeled Goat anti-chicken antibody (1:8000 dilution, Sigma Inc.) solution and incubated at 37°C for 1 hour. After another 5-time washing with PBS buffer, the immunoblot analysis was performed using Enhanced Chemiluminescence system (PIERCE Inc. ECL kit). Figure 11 shows that 12 days post DNA vaccination, chicken anti-HBx antibody reached the detectable level.

6.10. Map of (pImmo) used for immortalizing chicken B cells

As human B cells can be immortalized by EBV infection and mouse B cells can be immortalized directly with transfection of oncogenes, such as mutant *p53* and *Ras* oncogenes. Chicken B cells are selected for immortalization with chicken specific oncogene(s) using retroviral vectors transduction system, especially the lantiviral vector system which has the ability to infect the quiescence cells. The ASV (Avian Sarcoma Virus) based vector has been widely used in transforming chicken cells (Kaplitt et al., *Viral Vectors*, Academic Press, 1995). This section describes the construction of an HIV-based vector containing chicken mutant *p53* or *Ras* gene fragment, which can be used for chicken B cell immobilization.

Detailed design for constructing a new lantiviral vector based chicken B cell immortalization vector is described here. *HIV-1* based lantiviral vector (Naldini et al., *Science*, 1996, 272:263-268) is used as a starting material for the new vector construction. Chicken mutant *p53* oncogene is PCR amplified using following two oligos: Cp53-1 (5'ATGGCGGAGGAGATGGAACCA3') (SEQ ID NO:18) and Cp53-2 (5'TCAGTCCGAGCCTTTTTGCAGCAG 3') (SEQ ID NO:19) (Soussi et al., *Nucleic Acids Research*, 1988, 16:11383). The full-length chicken mutant *p53* oncogene is gel purified and inserted into pT7 Bleu(R) vector (Novagen Inc.) to generate the construct pT7-p53. 400 bp Cap-independent translation enhance (CITE) DNA fragment is PCR amplified from pCITE-5b(+) plasmid (Novagen Inc.; Parks et al., *J. Virol.*, 1986, 60:376-384) and inserted into pT7-p53 vector in the downstream of *p53* oncogene to produce the pT7-p53-CITE vector. Using two oligos, C-Ras-1 (5'ATGACCGAGTACAAGCTG3') (SEQ ID NO:20) and C-Ras-2 (5'TCACGATATCACGCATTTACAG 3') (SEQ ID NO:21), the chicken *Ras* oncogene is amplified by PCR and the *Ras* oncogene DNA fragment is inserted into pT7-p53-CITE to generate the dual oncogene expression vector: pT7-p53-CITE-Ras.

By digesting with HindIII-XhoI, LacZ gene DNA fragment in HIV-1 vector is replaced by chicken mutant p53-CITE-Ras oncogene DNA fragments to generate expression vector pHIV-1-Ch-p53-Ras. By linking two oncogenes with the CITE DNA fragment, the oncogenes' expression is driven under a single CMV promoter.

HIV-1 virus host cell specificity problem was overcome by pseudotyping with the G protein of vesicular stomatitis virus (*VSV-G*). To test this lantiviral vector's transduction efficiency, the experiments to generate the *HIV-1* based lantiviral vector transfection stock was performed as the following. Five ug pCMV-VSV-G plasmid, 5 ug *HIV-1* help plasmid pCMV*R9 and 10 expression vector pHIV-lacZ were mixed and transfected into $1x10^6$ 293 cells using calcium precipitation procedure (Promega Inc.). In 100 mm cell culture dish, $1x10^6$ 293 cells obtained from ATCC were seeded and cultured at 37°C, in 5% CO_2 incubator with 10% FCS supplemented DMEM medium in standard cell

culture environments. Four hours before transfection, pre-warmed 10% FCS medium was changed. Twelve hours after transfection, the 10 ml fresh pre-warmed medium were changed and 48 hours later, supernatant was collected and mixed with same volume of FCS to store the viral stock sample at –80°C. Alternatively, the viral stock is generated according to the protocol described in Chen et al., *Proc. Natl. Acad. Sci.*, 1996, 93:10057-10062).

The transduction of lantiviral vector for chicken cells was tested as the following. $5X10^5$ SL-29 cells (Chicken Embryo fibroblast cells obtained from ATCC) were seeded in 10 ml culture dish with MEM medium supplemented with 5% FCS. Next day, 100 ul serially diluted HIV-1-LacZ viral stock solution were added into SL-29 culture and incubated for 48 hours. All samples were operated in duplicate to control the variation. After washing cells once with PBS buffer, transduced cells were fixed with 0.25% (v/v in PBS) glutaraldehyde solution for 15 minutes, and stained with X-Gal solution (1 mg/ml X-Gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$-3X H_2O, 5 mM $K_3Fe(CN)_6$) for 2 hours at 37°C. By counting blue-stained cells, the pseudotyped lantiviral stock title in this experiment was determined to be 1.32×10^5 /ml.

Spleen B cells of DNA vaccinated chickens are immortalized as the following. Chicken spleen cells are collected and purified using Hypaque-Density Ficoll Gradient procedure (Sigma Inc.). After washing three times with PBS buffer, $1X10^5$ mixed B cells are seeded in a six well plate in 1 ml MEM-10% FCS medium and directly mixed with 2 ml viral stock solution overnight. Preferably the viral stock supernatant is treated with 5 mM dNTP and 2 mM spermidine at 37°C for 2 hours to enhance the viral infectivity (Zhang et al., *J. Virology*, 1995, 69:3929-3932). Four ug/ml polybrene (Sigma Inc) is also added into B cell culture to enhance the viral transduction efficiency during the viral/cell incubation. After B cells are incubated with the viral solution at 37°C overnight, B cells are diluted into the single well culture (10 cells/well) which contains feeding cells in the 96 well plate (400-500 chicken B cells/well irradiated with 20 Gays). The transformed cells are incubated for two to three weeks, the grown cell supernatant are first tested for the production of

IgY antibody (Davis, Ed., *Methods in Molecular Biology*, Monoclonal Antibody Protocols, 1995, Human Press), or screened for specific antigen binding IgY using ELISA as described above.

6.11. Determining housekeeping gene occurrence rate in the non-normalized human liver-derived cDNA library

In this experiment, the house-keeping gene occurrence rate in the single round DNA sequencing was detected. Two liver specific cDNA libraries were purchased from Invitrogen Inc. (Carlsbad, CA), catalog #: A550-39 and Clontech Inc (Palo Alto, CA), catalog #: HL400 2A2, respectively. After transferring 1 ul of the library stock to 500 ul LB medium, 10 ul were used to spray the LB plate which contains the selection antibiotic. After incubating the plate at 37°C overnight, from each of the cDNA library, 300 individual clones were picked up and cultured in 3 ml LB with shaking overnight. Each plasmid was prepared using Quigen Tip20 kit and 1 ug plasmid DNA was sequenced using ABI377 automatic DNA sequencing system with primer suggested by the library manufacturer. The sequencing data were analyzed by blasting sequence data comparison against GenBank database. The data were summarized in Table 1.

Table 1. Redundant Transcripts in Human Liver cDNA Library

Gene Name	Frequency
NADH-dehydrogenase Chain	7.2
Albumin	4.8
Actin	4.0
ATPase	4.0
α-Tubulin	3.2
Cytochrome Oxydase Chain	2.1
Elongation Factor 1α	1.0
Myosin LIght Chain	1.0
Aldolase	0.8

6.12. The prototype structure of antibody-chips

After stimulated by a specific antigen, each host B cell generates a specific antibody, which either binds to antigen specific sequential domain or conformational structure domain. A polyclonal antibody binds to a specific antigen through multiple binding sites. The antibody-chip comprising groups of specific antibodies on solid matrix support can be used to capture the free target protein (antigens) in a protein sample solution. After washing steps, the same group of antibody which is conjugated with an enzyme, such as HRP, or a detectable marker such as fluorescence dye (FITC or C3) can be used to further bind those captured antigen because of multiple binding domains of polyclonal antibodies, and to determine binding signal density with substrate of the enzyme such as ECL system or laser emission system (flowcytometer). In contrast, if the above capturing and labelled-binding steps are carried out using a monoclonal antibody, the binding efficiency is very low due to the single binding domain of the monoclonal antibody to the specific antigen. Although this problem could theoretically be overcome by using two monoclonal antibodies for every single antigen, the characterization of each different monoclonal antibody is extremely time consuming and hardly be practical.

Using AMIGAP of the present invention, one can generate multiple antibodies to unknown proteins or functionally undefined proteins. After purification of each IgY antibody, one can divide each of the antibody into two fractions and label one of the fraction with biotin (PIERCE Inc. IL. EZ-Link Biotinylating Reagents). Hundreds or thousands specific unlabeled antibodies are individually and randomly spotted on two identical solid support matrix (*e.g.*, 1 ug of each of antibody per spot on PVDF membrane or marked individual glass bead; and each spot or bead represents one known antibody). The spotted matrix is blocked with 5% BSA-PBS buffer to reduce the non-specific binding background. Specific group of antibodies, such as the antibodies targeting cell-cycle specific regulatory proteins or G-coupled receptor family proteins, can be used. The antibody-chip can be air-dried and stored at 4°C in the sealed plastic

bag for several months. Before performing the experiments, the antibody-chip can be activated by wetting the chip in PBS buffer for 30 minutes.

The system described here can be used in comparing the target protein expression in two samples, such as liver tumor cells vis-a-vis normal liver cells or human lung cancer cells treated with anti-cancer drug vis-a-vis untreated control cells. For preparing the protein sample, two target cell samples or tissues can be lysed by gentle detergents in PBS solution or freeze and thaw method (Sambrook et al., *Molecular Cloning*, Second Edition. Plainview, N.Y. Cold Spring Harbor Press, 1988). Same number of cells can be used for protein expression comparison. Alternatively, cell lysate sample are measured with its protein concentration first and then equal amount of protein sample are loaded onto the antibody-chips. Usually, $1X10^6$ cells per lysate sample or 50–100 ug proteins are used for each assay.

Each of protein samples is added to those identical antibody-chip and incubated at 37°C for 2 hours with slow shaking. After docking antibody specifically bind to its target antigen, simple washing steps is used to remove the un-captured cellular proteins. Antibody-chips are washed with PBS solution for four times, 15 – 30 minutes per washing.

As cellular target proteins are captured by membrane-bound docking antibodies, functioning as sandwich fashion, those captured target proteins are detected by mixture solution of biotin labeled antibodies which corresponds to each of the originally spotted antibodies and incubated 37°C for 2 hours. The signal density of the captured biotin-labeled antibodies is associated with the level of docking cellular protein level (antigen). Further quantification of captured biotin-labelled antibodies shows the antigen expression level in this assay. After washing 5 times in PBS buffer, the non-captured biotin-labeled antibodies on the antibody-chips are removed. Finally, avidin conjugated HRP (Sigma Inc. 1:8000 dilution in PBS with 2% BSA) is added and incubated for 15 minuets. After washing the antibody-chips with PBS solution for six times and soaking the antibody-chip with ECL substrate solution (PIERCE Inc. IL), the chip is exposed to X-ray film.

The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are intended to fall within the scope of the appended claims.

Various references are cited herein, the disclosures of which are incorporated herein by reference in their entireties.

What is claimed is:

1. A process for producing antibodies to an antigen in an avian species, which comprises:

1) delivering to said avian species a DNA sequence encoding said antigen operatively linked to a promoter, said promoter being capable of directing expression of said antigen in said avian species, or a mRNA sequence encoding said antigen, in an amount sufficient to induce detectable production of said antibodies to said antigen; and

2) recovering said antibodies from said avian species.

2. The process of claim 1, wherein the avian species is selected from the group consisting of a chicken, a turkey, a duck and a goose.

3. The process of claim 2, wherein the avian species is a chicken.

4. The process of claim 1, wherein the DNA or mRNA sequence is delivered directly to a tissue of the avian species.

5. The process of claim 4, wherein the tissue is muscle.

6. The process of claim 4, wherein the tissue is skin.

7. The process of claim 4, wherein the tissue is mucous membrane.

8. The process of claim 4, wherein the DNA or mRNA sequence is delivered by injection, by gene gun technology or by lipid mediated delivery technology.

9. The process of claim 1, wherein the DNA or mRNA sequence is delivered to a cell of the avian species and said cell containing the DNA or mRNA sequence is delivered to a suitable tissue of the avian species.

10. The process of claim 9, wherein the cell is selected from the group consisting of a blood cell and a spleen B cell.

11. The process of claim 9, wherein the DNA or mRNA sequence is delivered to the cell by a method selected from the group consisting of $Ca_3(PO_4)_2$-DNA transection, DEAE dextran-DNA transfection, electroporation, transfection using "LIPOFECTIN"™ reagent, gene gun technology and viral gene delivery system.

12. The process of claim 1, wherein a DNA sequence encoding the antigen operatively linked to a promoter is delivered.

13. The process of claim 12, wherein the DNA sequence is in the form of a plasmid.

14. The process of claim 12, wherein the promoter is an endogenous promoter of the avian species.

15. The process of claim 12, wherein the promoter is a viral promoter which is capable of directing expression of the antigen in the avian species.

16. The process of claim 15, wherein the viral promoter is selected from the group consisting of RSV LTR, MPSV LTR, SV40 IEP, CMV IEP, spleen necrosis virus LTR (SNV LTR) and metallothionein promoter.

17. The process of claim 12, wherein the DNA sequence further comprises a sequence that directs secretion of the encoded antigen in the avian species.

18. The process of claim 17, wherein the secretion-directing sequence is a leader sequence.

19. The process of claim 18, wherein the leader sequence is an endogenous leader sequence of the avian species.

20. The process of claim 18, wherein the leader sequence is selected from the group consisting the leader sequence of chicken IgY, chicken SPARC, chicken serum albumin and chicken tissue-type plasminogen activator (tPA).

21. The process of claim 18, wherein the leader sequence is selected from the group consisting of the leader sequence of IL-1, CD4 and MHC.

22. The process of claim 1, wherein a mRNA sequence encoding the antigen is delivered.

23. The process of claim 1, wherein the avian species is a chicken and the antibodies are recovered from egg yolk of the chicken.

24. The process of claim 23, wherein the antibodies are purified from the egg yolk by ammonium sulfate precipitation, by polyethylene glycol 6000 precipitation or by caprylic acid precipitation.

25. The process of claim 1, wherein the avian species is a chicken and the antibodies are recovered from the antibody-producing B cells of the chicken.

26. The process of claim 1, wherein the antigen is a secreted protein or peptide.

27. A process for producing a monoclonal antibody to an antigen in a chicken, which comprises:

1) delivering to said chicken a DNA sequence encoding said antigen operatively linked to a promoter, said promoter being capable of directing expression of said antigen in said chicken, or a mRNA sequence encoding said antigen, in a amount sufficient to induce detectable production of said antibodies to said antigen;

2) removing at least a portion of antibody-producing cells from said chicken;

3) immortalizing said removed antibody-producing cells;

4) propagating said immortalized antibody-producing cells; and

5) harvesting said monoclonal antibody produced by said immortalized antibody-producing cells.

28. The process of claim 27, wherein the antibody-producing cells being removed in step 2) are chicken spleen B cells.

29. The process of claim 28, wherein the chicken spleen B cells are immortalized by fusing with cells of a chicken B lymphoblastoid cell line.

30. The process of claim 29, wherein the chicken B lymphoblastoid cell line is selected from the group consisting of HU3R27, HU3R27N and R27H4.

31. The process of claim 28, wherein the chicken spleen B cells are immortalized by oncogene transformation.

32. The process of claim 31, wherein the oncogene used in transformation is mutant chicken *p53* oncogene or *Ras* oncogene.

33. A vector for expressing genes in avian and bacterial cells, which comprises the plasmid depicted in Figure 3A or Figure 3C.

34. A process for determining the proteomics profile of a set of pre-selected DNA sequences isolated from a bio-sample, which comprises:

1) cloning each of said DNA sequences into a dual-expression vector that is capable of expressing said DNA sequences in avian and bacterial cells;

2) delivering said DNA sequence in said dual-expression vector formed in step 1) to an avian species in an amount sufficient to induce detectable production of antibodies to an antigen encoded by said DNA sequence, and recovering said antibodies from said avian species;

3) delivering said DNA sequence, which is delivered to said avian species in step 2), to bacterial cells, and recovering proteins or peptides encoded by said DNA sequence from said bacterial cells;

4) conducting immunoreactions between said antibodies recovered in step 2) with said proteins or peptides recovered from step 3) to characterize the immunospecificity of said antibodies; and

5) conducting immunoreactions between said antibodies recovered in step 2) with said bio-samples to determine the proteomics profile of said set of pre-selected DNA sequences.

35. The process of claim 34, wherein the set of pre-selected DNA sequences is a cDNA library.

36. The process of claim 34, wherein the bio-sample is of human origin.

37. The process of claim 34, wherein the dual-expression vector is the plasmid depicted in Figure 3A or Figure 3C.

38. The process of claim 34, wherein the avian species is selected from the group consisting of a chicken, a turkey, a duck and a goose.

39. The process of claim 38, wherein the avian species is a chicken.

40. The process of claim 34, wherein the DNA sequence is delivered directly to a tissue of the avian species.

41. The process of claim 40, wherein the tissue is muscle.

42. The process of claim 40, wherein the tissue is skin.

43. The process of claim 40, wherein the tissue is mucous membrane.

44. The process of claim 40, wherein the DNA sequence is delivered by injection, by gene gun technology or by lipid mediated delivery technology.

45. The process of claim 34, wherein the DNA sequence is delivered to a cell of the avian species and said cell containing the DNA sequence is delivered to a suitable tissue of the avian species.

46. The process of claim 45, wherein the cell is selected from the group consisting of a blood cell and a spleen B cell.

47. The process of claim 44, wherein the DNA sequence is delivered to the cell by a method selected from the group consisting of $Ca_3(PO_4)_2$-DNA transection, DEAE dextran-DNA transfection,

electroporation, transfection using "LIPOFECTIN"™ reagent, gene gun technology and viral gene delivery system.

48. The process of claim 34, wherein the avian species is a chicken and in step 2) the antibodies are recovered from egg yolk of the chicken.

49. The process of claim 48, wherein the antibodies are purified from the egg yolk by ammonium sulfate precipitation, by polyethylene glycol 6000 precipitation or by caprylic acid precipitation.

50. The process of claim 34, wherein the avian species is a chicken and in step 2) the antibodies are recovered from the antibody-producing B cells of the chicken.

51. The process of claim 34, wherein the bacterial cells are *E. coli* cells.

52. The process of claim 34, wherein the immunoreactions are assayed by immunoblotting, immunoprecipitation or *in situ* immunostaining.

53. The process of claim 34, wherein in step 5) the immunoreactions are conducted to determine the existence, quantity, subcellular location or tissue expression specificity of proteins or peptides encoded by the set of pre-selected DNA sequences in evaluating proteomics profile of the set of pre-selected DNA sequences in the bio-sample.

54. The process of claim 34, wherein the pre-selected DNA sequences is isolated from a physiologically normal bio-sample.

55. The process of claim 34, wherein the pre-selected DNA sequences is isolated from a physiologically abnormal bio-sample.

56. The process of claim 34, wherein the DNA sequence further comprises a sequence that directs secretion of the encoded antigen in the avian species.

57. The process of claim 56, wherein the secretion-directing sequence is a leader sequence.

58. The process of claim 57, wherein the leader sequence is an endogenous leader sequence of the avian species.

59. The process of claim 57, wherein the leader sequence is selected from the group consisting the leader sequence of chicken IgY, chicken SPARC, chicken serum albumin and chicken tissue-type plasminogen activator (tPA).

60. The process of claim 58, wherein the leader sequence is selected from the group consisting of the leader sequence of IL-1, CD4 and MHC.

61. The process of claim 35, wherein the cDNA library encodes secreted proteins or peptides in the bio-sample.

62. A process for identifying physiologically distinguishable markers associated with a physiologically abnormal bio-sample, which comprises:

1) determining proteomics profile of said physiologically abnormal bio-sample through the process of claim 34;

2) determining proteomics profile of a comparable physiologically normal bio-sample through the process of claim 34; and

3) comparing the proteomics profile obtained in step 1) with the proteomics profile obtained in step 2) to identify physiologically distinguishable markers associated with a physiologically abnormal bio-sample.

63. A vector for immortalizing chicken antibody-producing cells, which comprises the plasmid depicted in Figure 12.

DNA Clone Selection

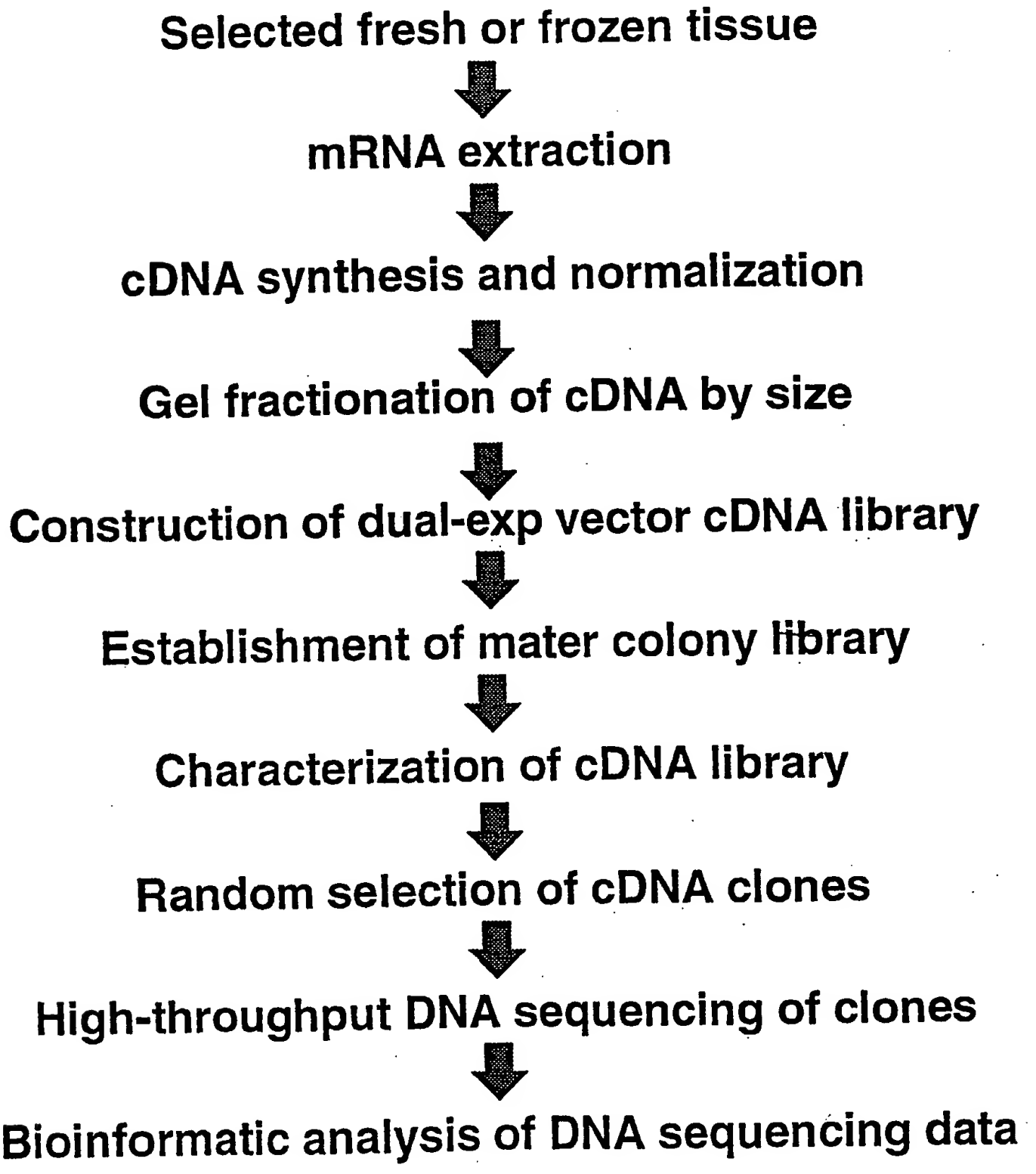


FIG. 1

Flow Chart of AMIGAP

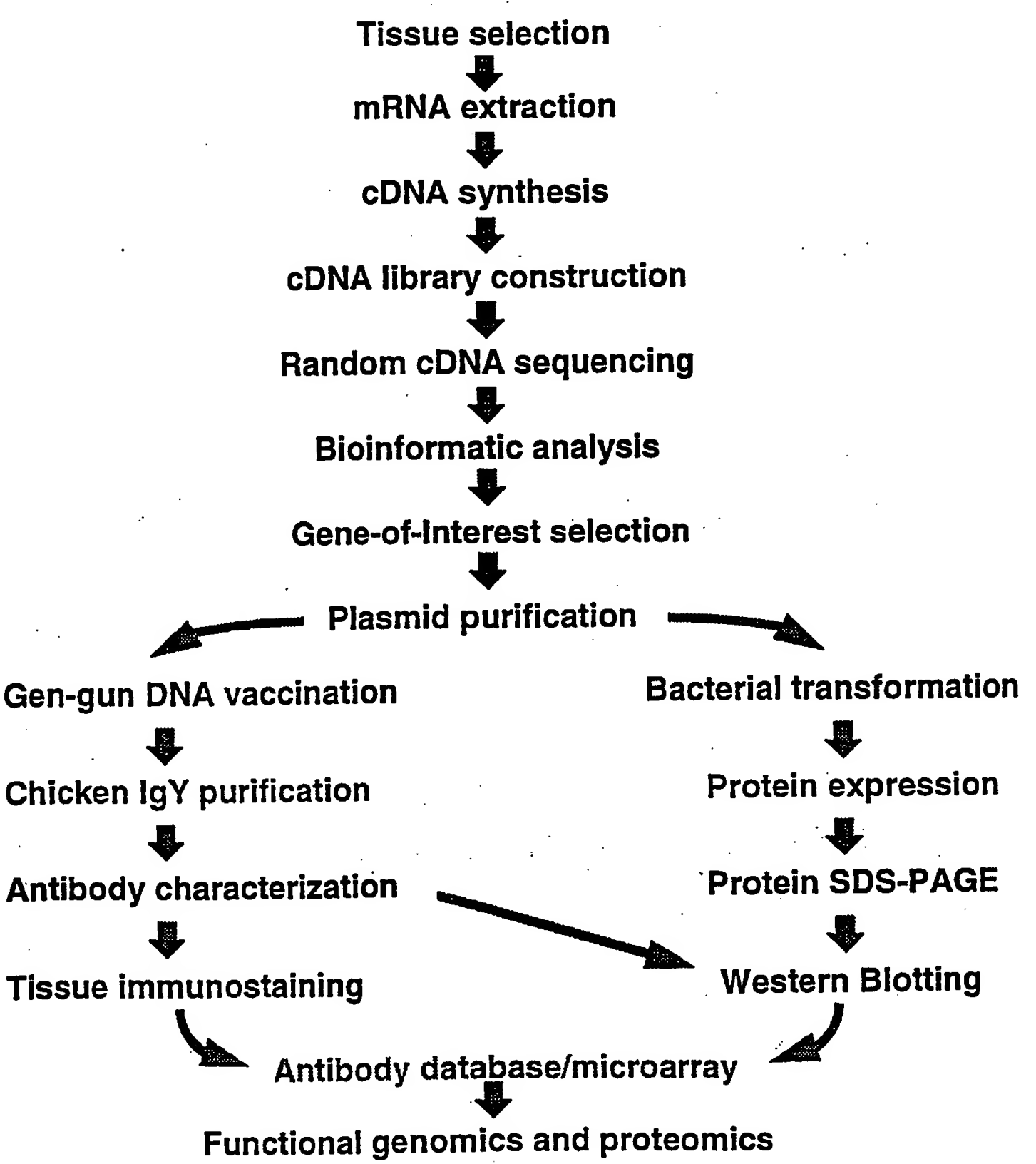
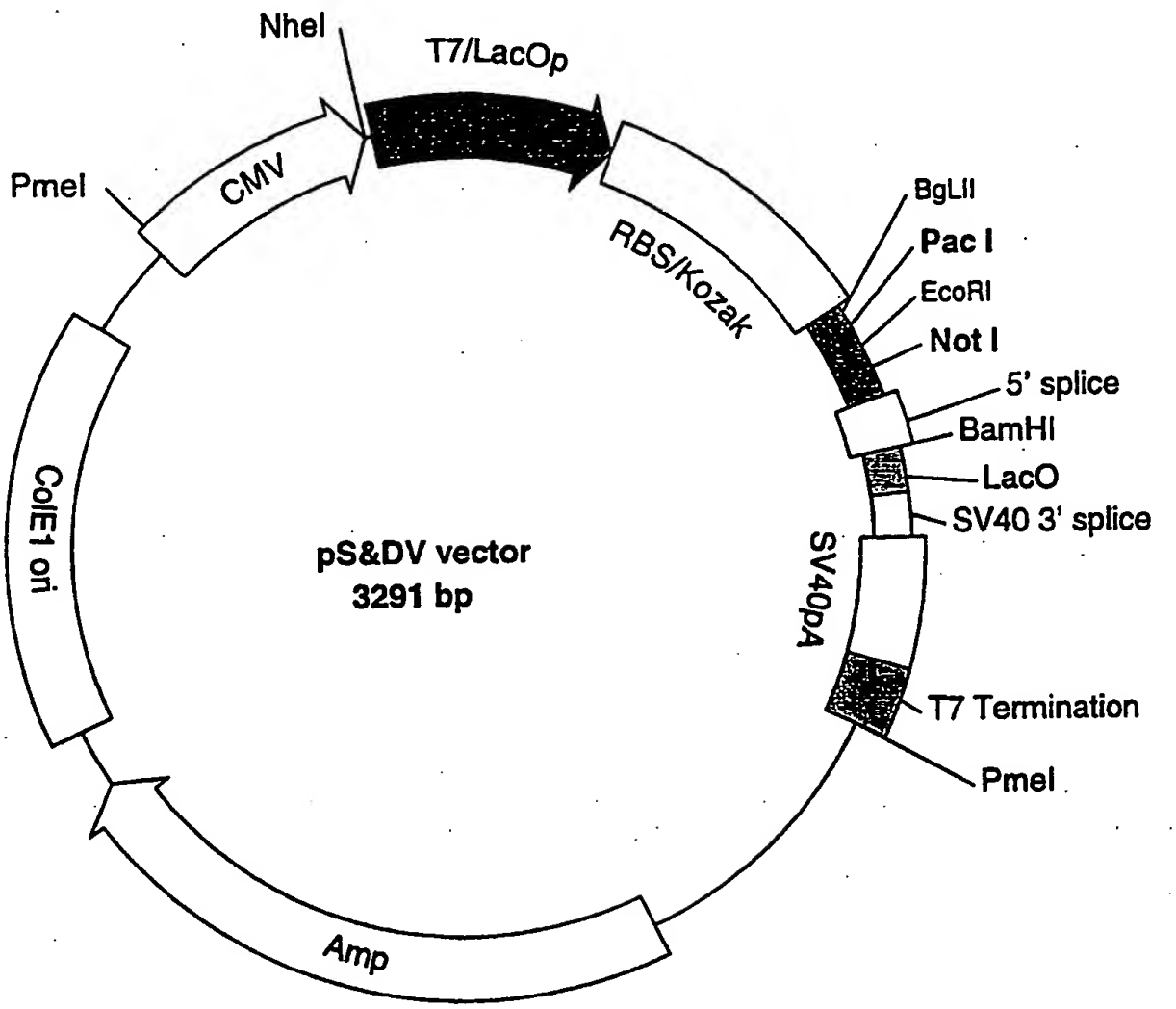


FIG. 2



Name of plasmid: pS&DV
Size of Vector: 3291 bp
Constructed by: Lingxun Duan. GenWay BioTech Inc

polilinker region DNA sequence:

ATG g *AGATC*<u>***TTAATTAA***</u> GAATTC <u>GCGGCCGC</u> TACCAGGTAAGTG

FIG. 3A

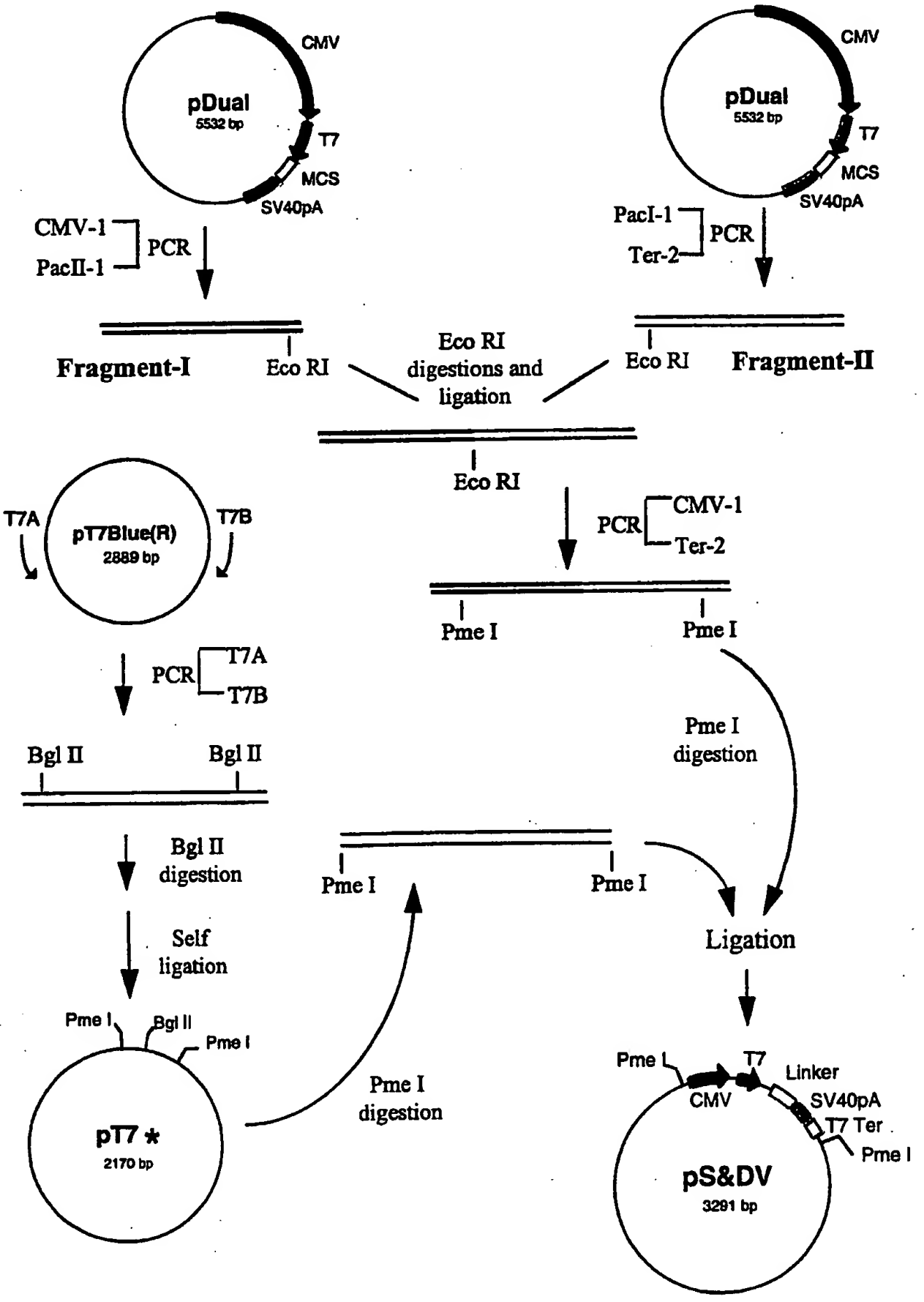
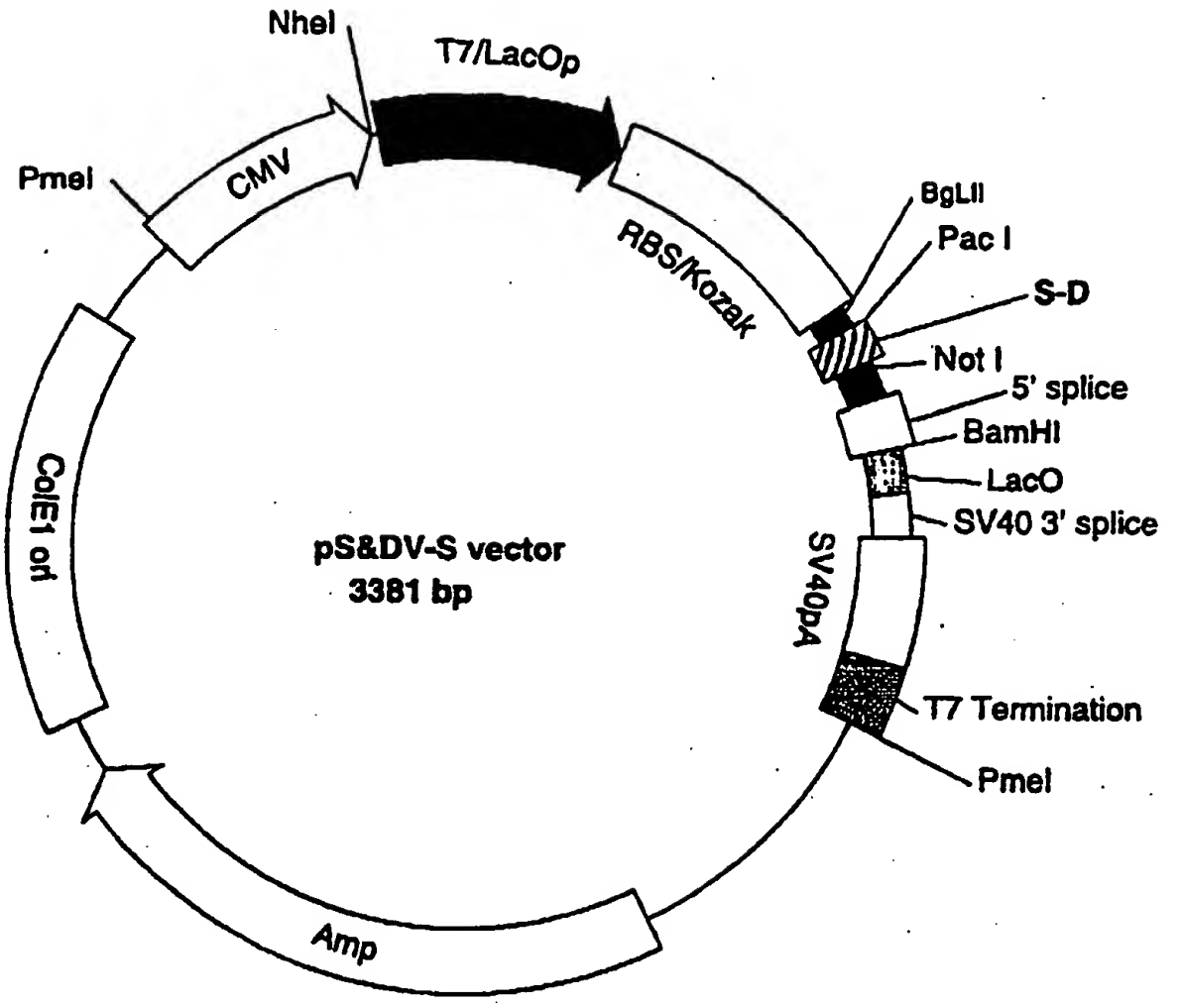


Figure 3B. Construction of Dual Expression Vector pS&DV



Name of plasmid: pS&DV-S
Size of Vector: 3381 bp
Constructed by: Lingxun Duan. GenWay BioTech Inc

S-D : chicken IgY Vh1 secreted domain:
MSPLVSSLLLLAALPRLMAA—inserted protein domain

polilinker region DNA sequence:

ATGAGCCCACTCGTCTCCTCCCTCCTGCTCCTGGCCGCCCTGCCAGGGCTGATG
GCG GCC ***TTAATTAA*** GAATTC **GCGGCCGC** TACCAGGTAAGTG

FIG. 3C

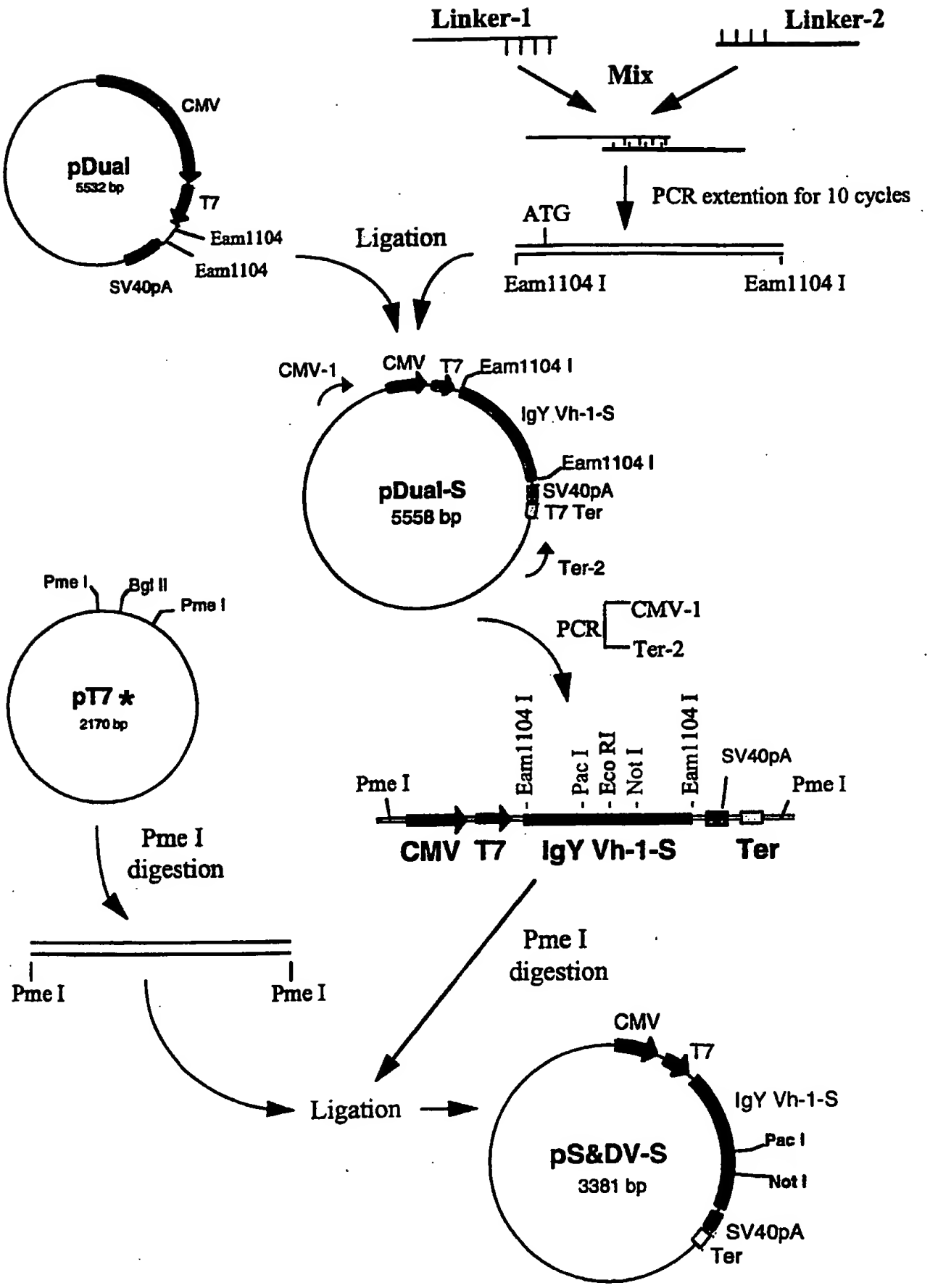


Figure 3D. Construction of Dual Expression Vector pS&DV-S

Immune Response Elicited by DNA

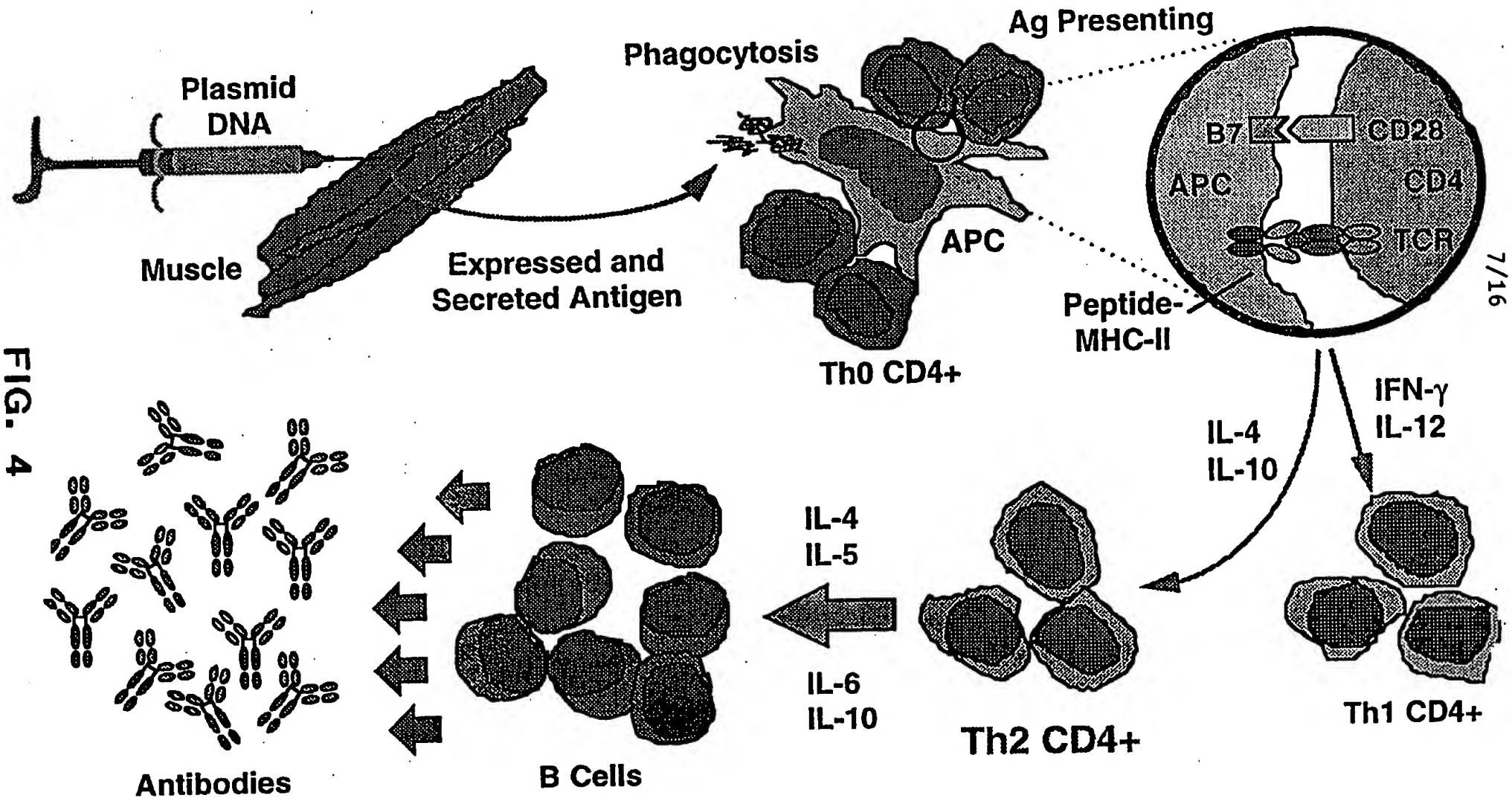
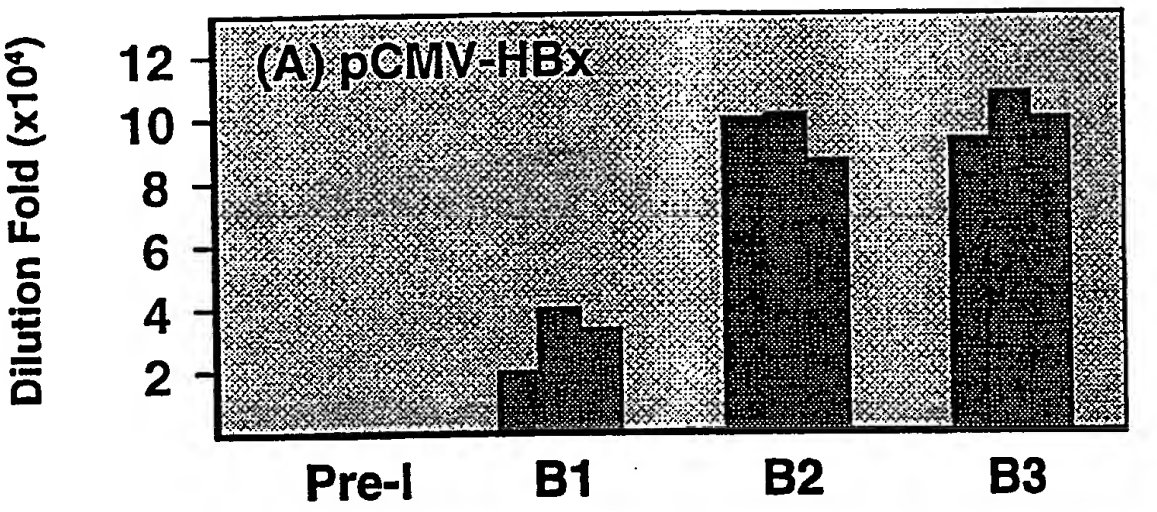
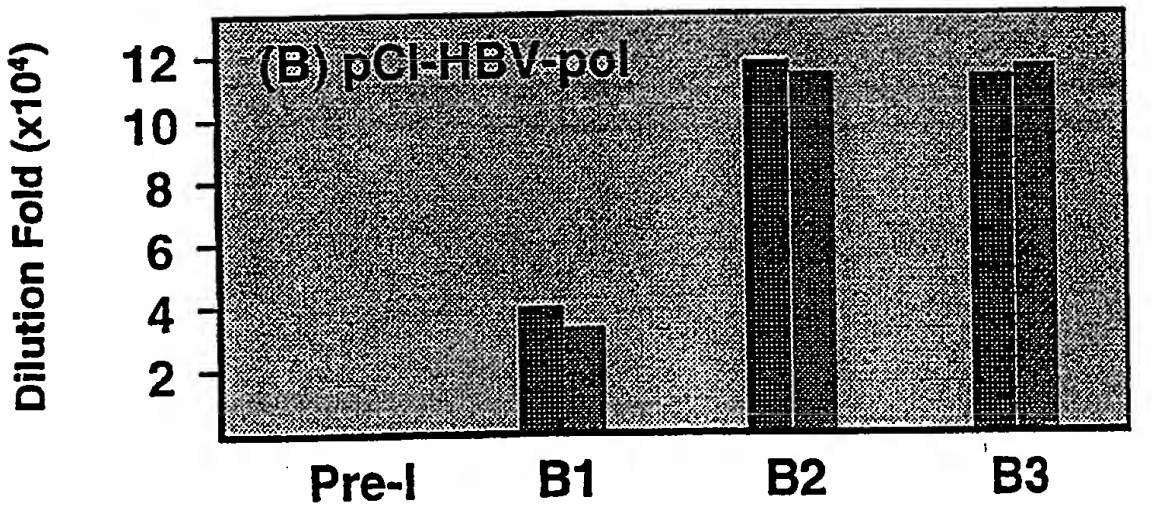


FIG. 4

ELISA Titerring of Antibodies





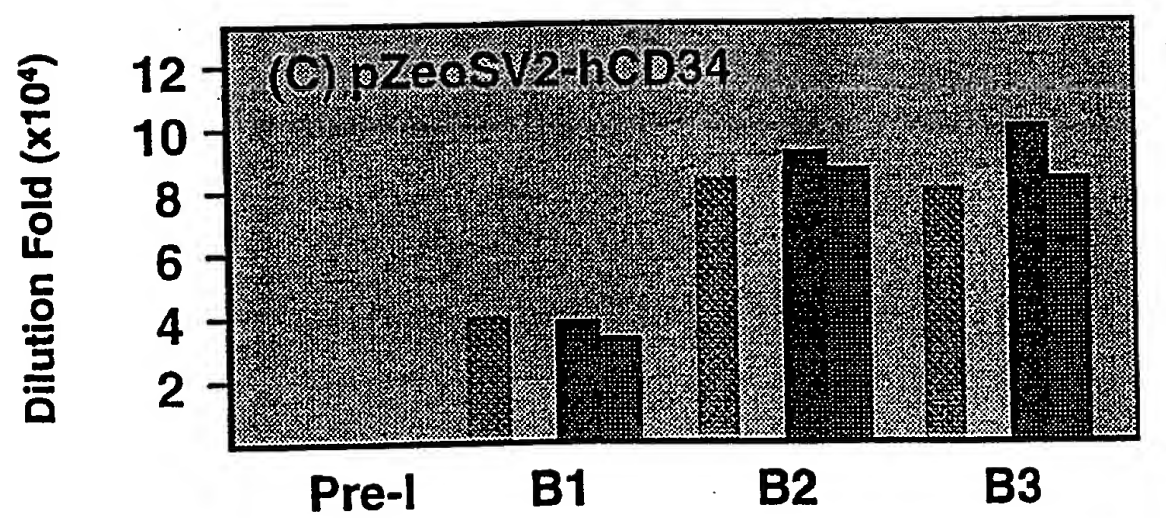
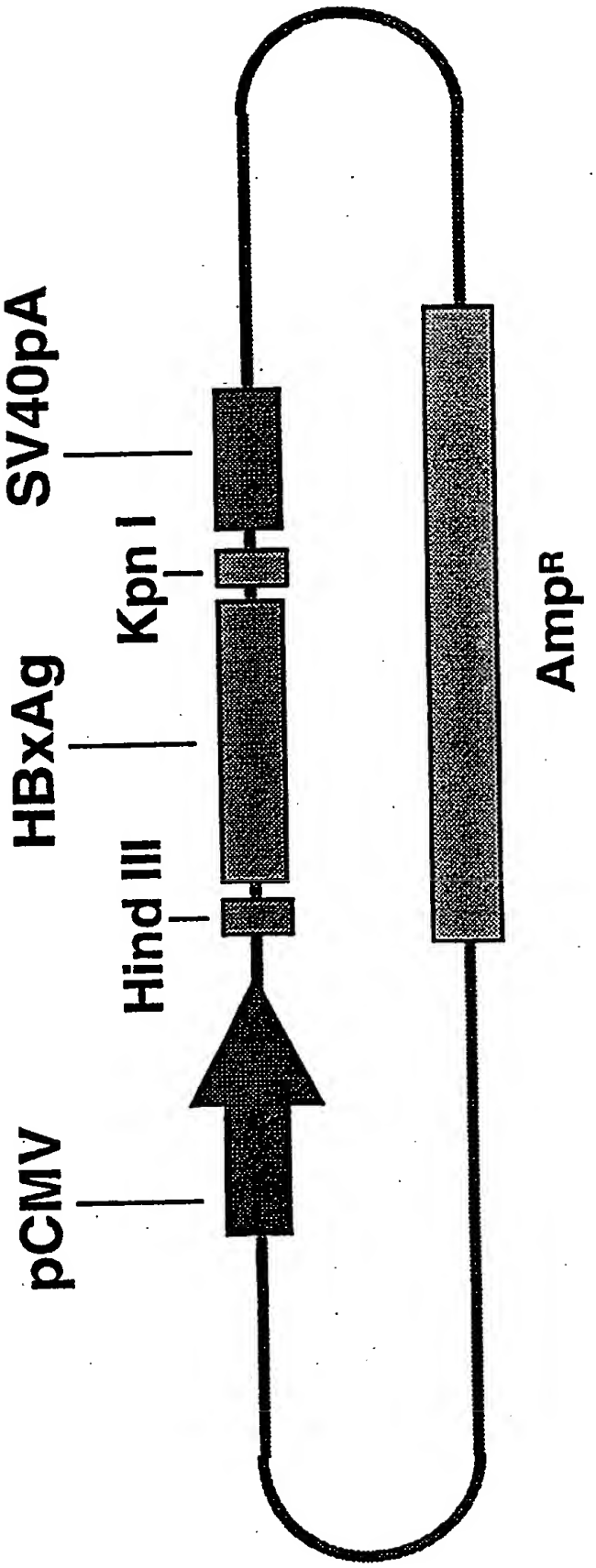


FIG. 5

Plasmid Map of pCMV-HBx



HBxAg: HBx Antigen

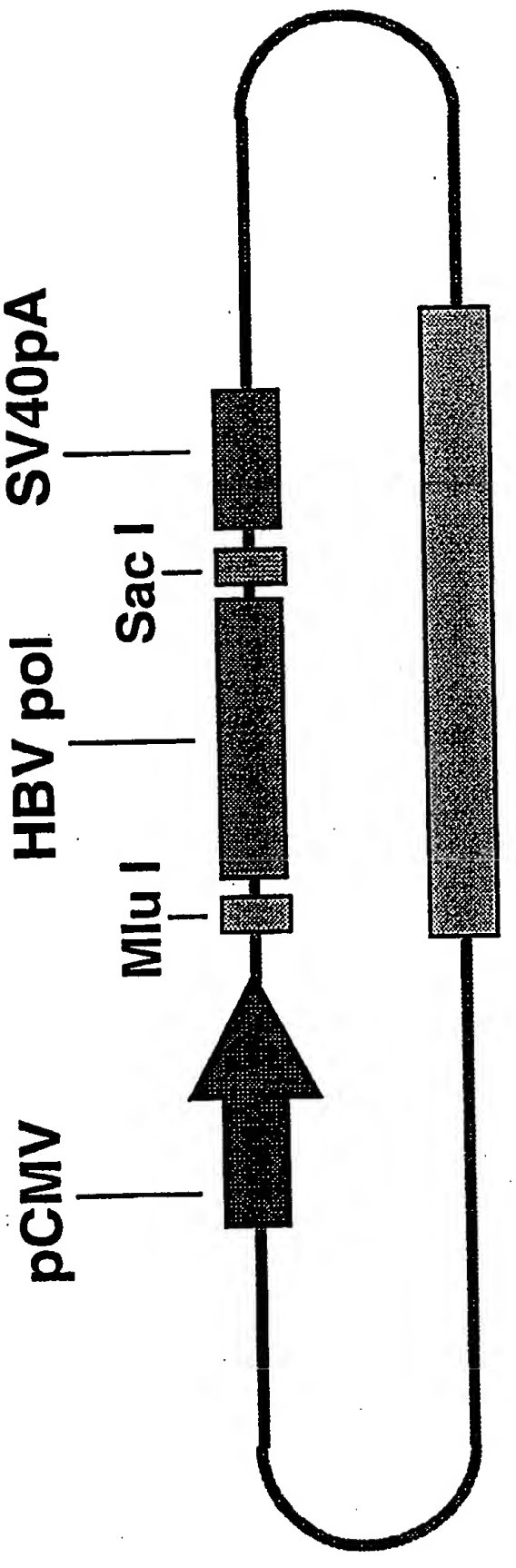
SV40pA: SV40 Polyadenylation Signal

pCMV: CMV Promoter

Amp^R: Ampicilin Resistant Gene

FIG. 6

Plasmid Map of pCI-HBV-pol



HBV pol: HBV Polymerase Gene

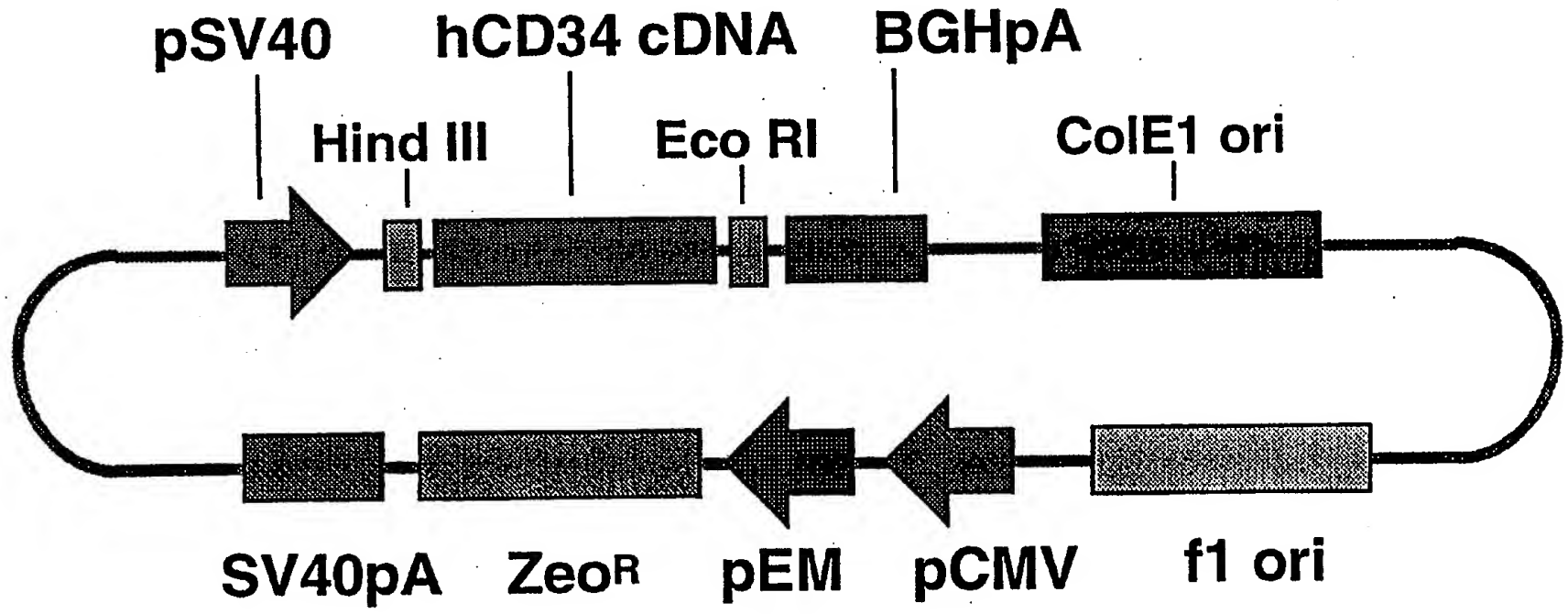
SV40pA: SV40 Polyadenylation Signal

pCMV: CMV Promoter

AmpR: Ampicilin Resistant Gene

FIG. 7

Plasmid Map of pZeoSV2-hCD34



pSV40: SV40 Promoter
pEM: Synthetic EM-7 Promoter
pCMV: CMV Promoter
Zeo^{R}: Zeocin^{TM} Resistant Gene

BGHpA: Bovine GH ploy-A Signal
SV40pA: SV40 Polyadenylation Signal
ColE1 ori: Replication in E. Coli
f1 ori: For rescue of ssDNA

FIG. 8

PCT/US99/26843

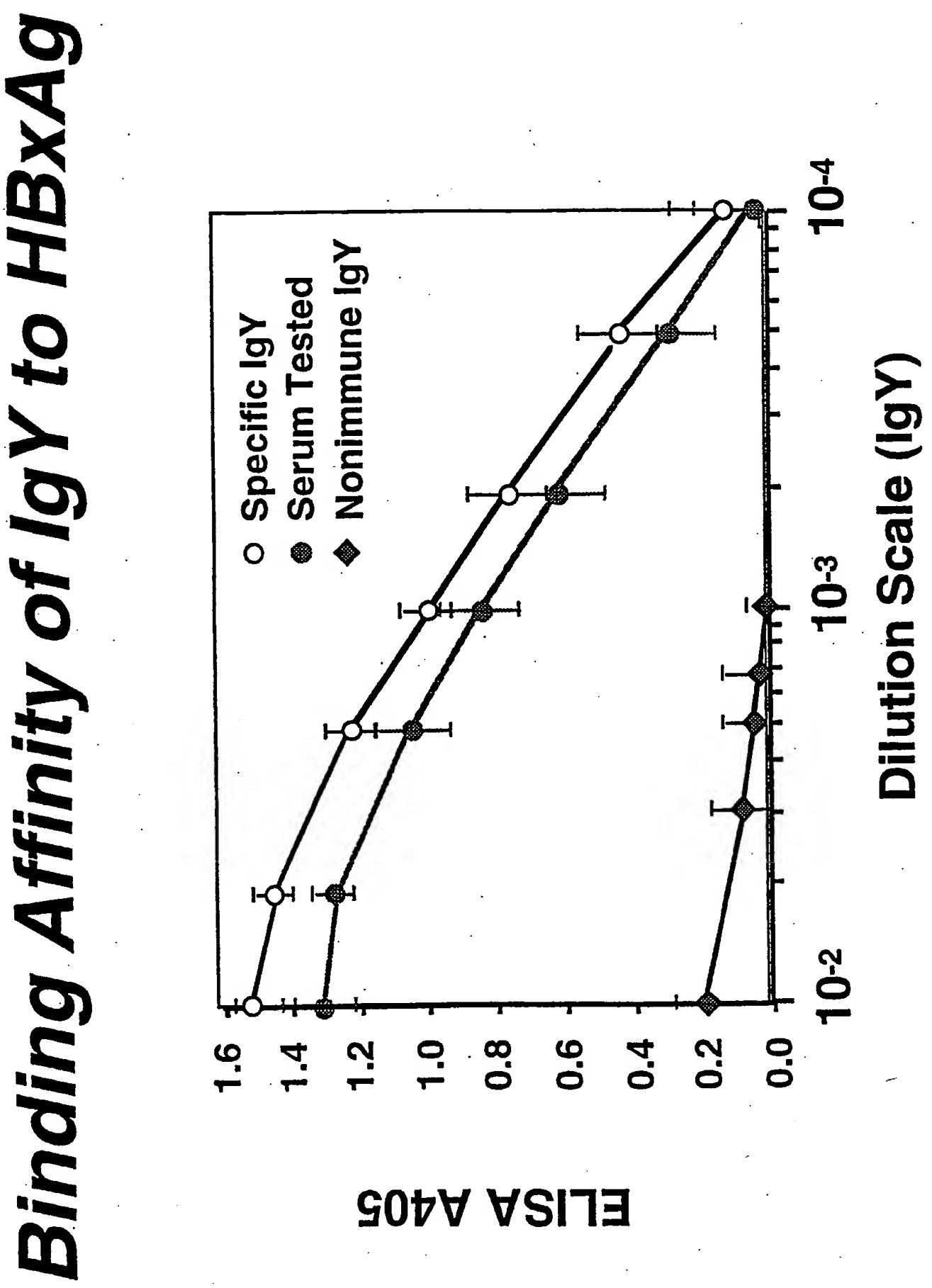

Binding Affinity of IgY to HBxAg
○ Specific IgY
● Serum Tested
◆ Nonimmune IgY
ELISA A405
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
10-2
10-3
10-4
Dilution Scale (IgY)

FIG. 9

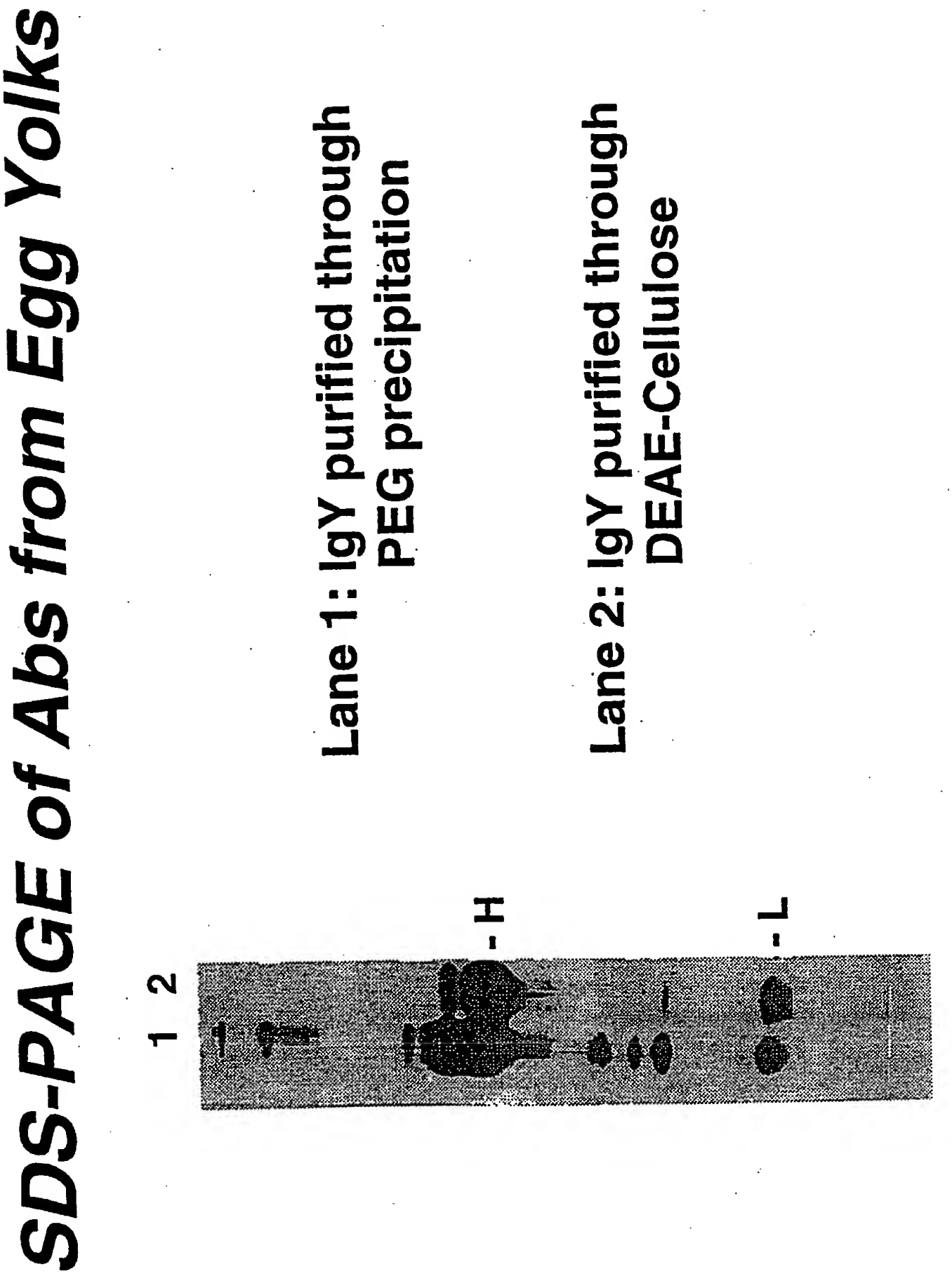

SDS-PAGE of Abs from Egg Yolks
Lane 1: IgY purified through PEG precipitation
Lane 2: IgY purified through DEAE-Cellulose
1 2
H
L

FIG. 10

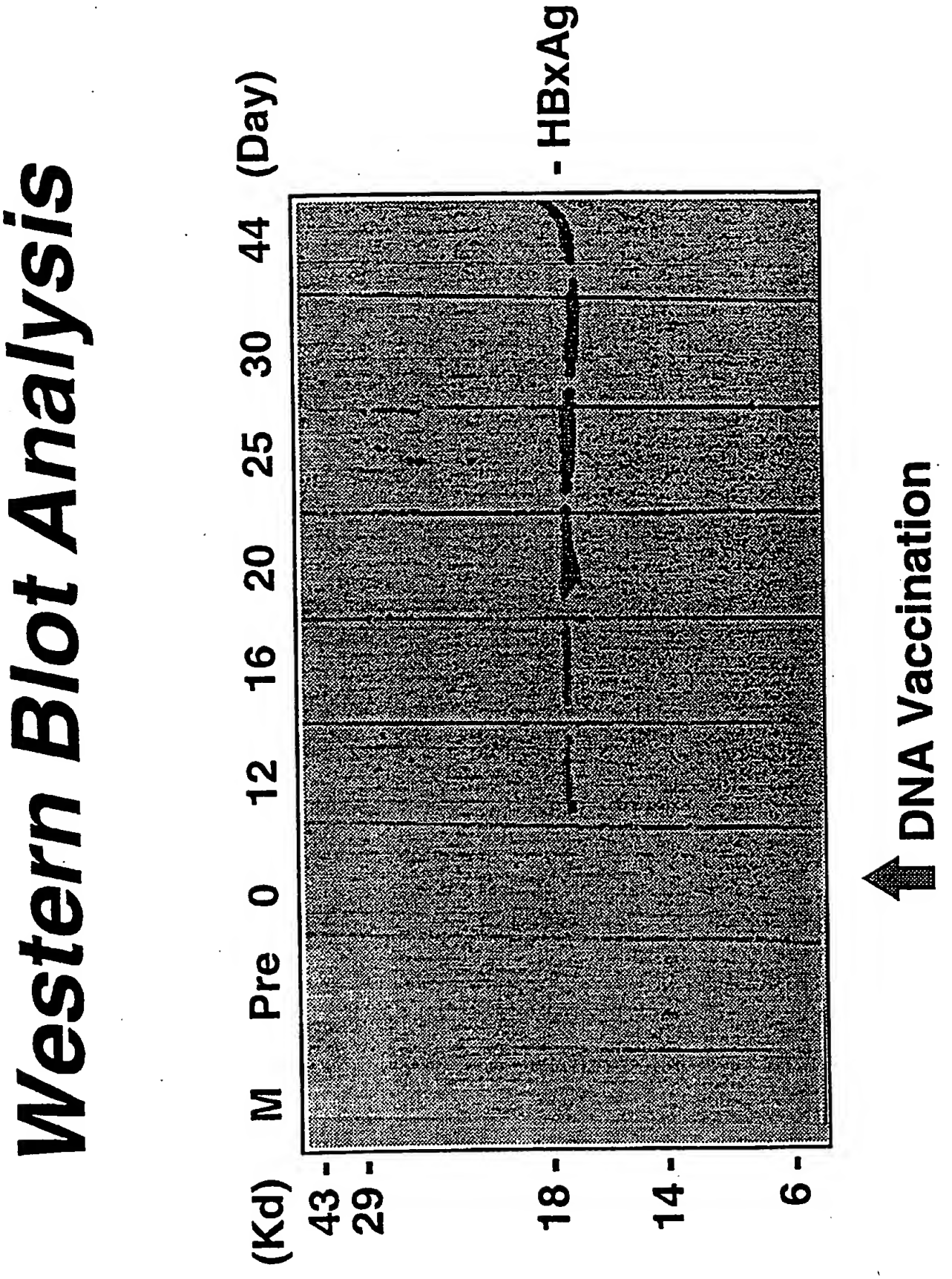
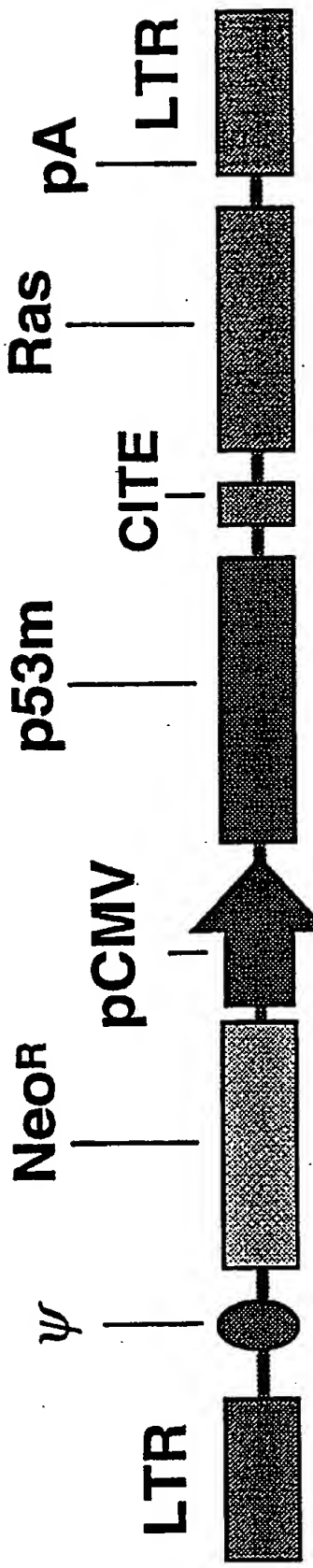

Western Blot Analysis
(Kd)
M
Pre
0
12
16
20
25
30
44
(Day)
- HBxAg
43 -
29 -
18 -
14 -
6 -
DNA Vaccination

FIG. 11

Genomic Map of plmmo Vector



pCMV: CMV Promoter
p53m: p53 mutant cDNA
Ras: Ras Oncogene
NeoR: Neomycin Resistant Gene
CITE: Cap-Independent Translation Enhancer
pA: Polyadenylation Signal
LTR: Long Terminal Repeats
ψ: Packaging Signal

FIG. 12

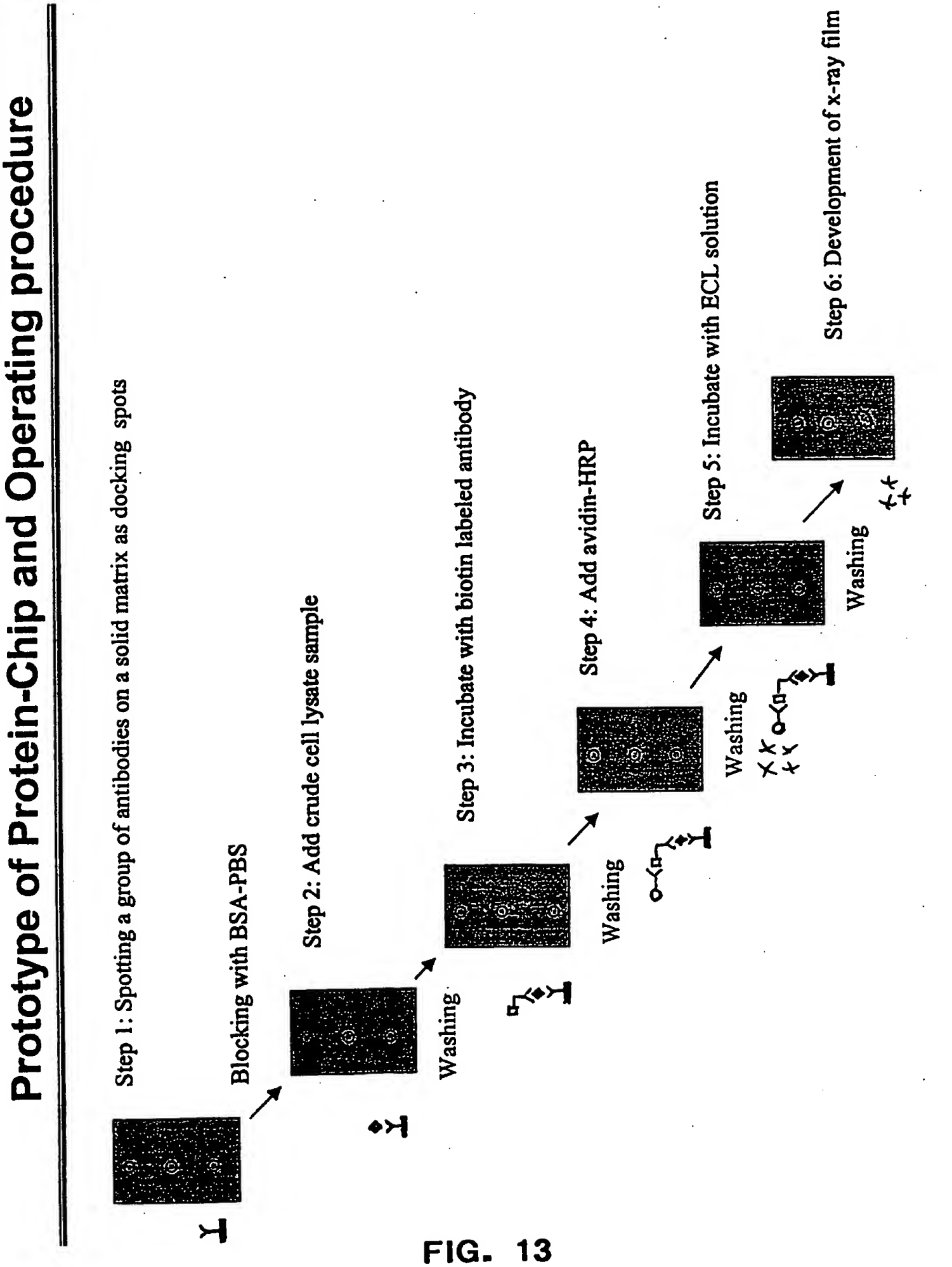

Prototype of Protein-Chip and Operating procedure
Step 1: Spotting a group of antibodies on a solid matrix as docking spots
Blocking with BSA-PBS
Step 2: Add crude cell lysate sample
Washing
Step 3: Incubate with biotin labeled antibody
Washing
Step 4: Add avidin-HRP
Washing
Step 5: Incubate with ECL solution
Step 6: Development of x-ray film

FIG. 13

SEQUENCE LISTING

<110> Duan, Linxun

<120> Methods and Vectors for Generating Antibodies Using Polynucleotide Vaccination in Avian Species

<130> 0003

<140>
<141>

<150> 60/108,487
<151> 1998-11-16

<160> 21

<170> PatentIn Ver. 2.0

<210> 1
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing pS&DV

<400> 1
caccctgaat tgactctctt tc 22

<210> 2
<211> 42
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing pS&DV

<400> 2
atatgaattc ttaattaaga tctccatggt ggcctctcct tc 42

<210> 3
<211> 35
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing pS&DV

<400> 3
cgcggaattc gcggccgcta ccaggtaagt gtacc 35

<210> 4
<211> 34
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing pS&DV

<400> 4
cgagtagttt aaacaaaaaa cccctcaagt cccg 34

<210> 5
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing pS&DV

<400> 5
agatctgttt aaaccaggtg gcacttttcg g 31

<210> 6
<211> 31
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing pS&DV

<400> 6
agatctgttt aaacagctgt ttcctgtgtg a 31

<210> 7
<211> 20
<212> PRT
<213> chicken IgY Vh1 signal domain

<400> 7
Met Ser Pro Leu Val Ser Ser Leu Leu Leu Ala Ala Leu Pro Gly
1 5 10 15

Leu Met Ala Ala
20

<210> 8
<211> 71
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing pS&DV

<400> 8
aaccctcttc catgagccca ctcgtctcct ccctcctgct cctggccgcc ctgccagggc 60
tgatggcggc c 71

<210> 9
<211> 62
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing pS&DV

<400> 9
cagatcctct tcgaagctag cggccgcgaa ttcttaatta aggccgccat cagccctggc 60
ag 62

<210> 10
<211> 32
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing pCMV-HBx

<400> 10
ggaagcttgc cgccatggct gctaggctgt gc 32

<210> 11
<211> 25

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing pCMV-HBx

<400> 11
gtggagacgg attagtacca tggcc 25

<210> 12
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing hepetitis B polymerase

<400> 12
aagagctcgc caccatggcc ctatcctatc aac 33

<210> 13
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing hepetitis B polymerase

<400> 13
tcaccttaag gtgttggaag gtggtttga 29

<210> 14
<211> 29
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing hepatitis B polymerase

<400> 14
ggccatgcag tggaattcca ctgccttcc 29

<210> 15
<211> 30

<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing hepatitis B polymerase

<400> 15
aaccaagctt cacggtggtc tggatgcaac 30

<210> 16
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing hCD34 expression vector

<400> 16
gaaggatgct ggtccgcagg gg 22

<210> 17
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:oligo for constructing hCD34 expression vector

<400> 17
cacctagccg agtcacaatt cg 22

<210> 18
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Cp53-1

<400> 18
atggcggagg agatggaacc a 21

<210> 19
<211> 24
<212> DNA

<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:Cp53-2

<400> 19
tcagtccgag ccttttgca gcag 24

<210> 20
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:C-Ras-1

<400> 20
atgaccgagt acaagctg 18

<210> 21
<211> 22
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence:C-Ras-2

<400> 21
tcacgatatc acgcatttac ag 22

INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/US 99/26843

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K16/00 C07K16/02 A61K48/00 C12Q1/68 G01N33/577
C12N15/70 C12N15/85 C12N15/867

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 24268 A (ARTHUR WEBSTER) 27 October 1994 (1994-10-27) claims 1-12 page 10, line 18 - line 29	1-19, 22-26
Y		27-32
Y	NISHINAKA S ET AL: "Two chicken B cell lines resistant to ouabain for the production of chicken monoclonal antibodies." JOURNAL OF VETERINARY MEDICAL SCIENCE, vol. 58, no. 11, 1 January 1996 (1996-01-01), pages 1053-6, XP002061707 abstract	27-32

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 March 2000	20/04/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Le Flao, K

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In. ational Application No
PCT/US 99/26843

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	US 4 518 690 A (GUNTAKA R) 21 May 1985 (1985-05-21) claims 1-25	33
A	MARTINON F ET AL: "Induction of virus-specific cytotoxic T lymphocytes in vivo by liposome-entrapped mRNA." EUR. J. IMMUNOL., vol. 23, 1993, pages 1719-22, XP000618955 abstract	8-11,22
A	AKIYAMA T ET AL: "Tissue-specific expression of mouse tyrosinase gene in cultured chicken cells" EXPERIMENTAL CELL RESEARCH, vol. 214, no. 1, September 1994 (1994-09), pages 154-62, XP000877398 abstract	16
A	MCLAREN R ET AL: "The use of caprylic acid for the extraction of the immunoglobulin fraction from egg yolk of chickens immunised with ovine alpha-lactalbumin." JOURNAL OF IMMUNOLOGICAL METHODS, vol. 177, no. 1-2, 1994, pages 175-84, XP002132447 abstract	23,24
A	WO 98 22510 A (ARCH DEVELOPMENT CORPORATION) 28 May 1998 (1998-05-28) claims 1-26	1-63
T	BLACKSTOCK W ET AL: "Proteomics: quantitative and physical mapping of cellular proteins." TRENDS IN BIOTECHNOLOGY, vol. 17, no. 3, March 1999 (1999-03), pages 121-7, XP004157732 the whole document	34-62
P,X	WO 99 02188 A (THE ARIZONA BOARD OF REGETS) 21 January 1999 (1999-01-21) claims 1-20	1-4

INTERNATIONAL SEARCH REPORT

Information on patent family members

Int ational Application No
PCT/US 99/26843

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9424268 A	27-10-1994	AU 676042 B	27-02-1997
		AU 6499494 A	08-11-1994
		EP 0690912 A	10-01-1996
		JP 8508410 T	10-09-1996
		NZ 263772 A	20-12-1996
US 4518690 A	21-05-1985	NONE	
WO 9822510 A	28-05-1998	AU 5686698 A	10-06-1998
WO 9902188 A	21-01-1999	AU 8395498 A	08-02-1999